UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

Determining the prognostic significance of PI3K/Akt/mTOR and JAK/STAT5 signaling pathways in pediatric acute lymphoblastic leukemia using single-cell analysis

Mariana Lobato de Oliveira

Mestrado em Oncobiologia

Dezembro 2014

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

Determining the prognostic significance of PI3K/Akt/mTOR and JAK/STAT5 signaling pathways in pediatric acute lymphoblastic leukemia using single-cell analysis

Mariana Lobato de Oliveira

Tese orientada pelo Doutor João Pedro Taborda Barata

Mestrado em Oncobiologia

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 27 de Janeiro de 2015.

Acknowledgments/Agradecimentos

Gostaria, em primeiro lugar, de agradecer ao meu orientador, o Dr. João Taborda Barata, por me ter aceite como sua estudante de mestrado no seu laboratório e por me ter proporcionado um projecto difícil, mas acima de tudo desafiante. Obrigado por todo o suporte, motivação e conhecimentos facultados ao longo deste ano, bem como por todas as críticas e sugestões que ajudaram a melhorar este projecto.

Porque foi com ela que aprendi todas as técnicas necessárias ao desenvolvimento deste projecto, queria deixar um enorme agradecimento à Leila Martins. Obrigado por todo o tempo precioso que passaste comigo na bancada, e por todos os conselhos e dicas que me deste. Um grande beijinho para o Gustavo que, no final de contas, esteve connosco todos os dias durante os primeiros 3 meses de trabalho.

Como sem eles o tempo na minha segunda "casa" passaria mais devagar, não podia deixar de agradecer a todos os meus colegas de laboratório, passados e actuais, pelos bons momentos tanto de trabalho como de diversão. O meu obrigado à Rita Fragoso, Isabel Alcobia, Leonor Sarmento, Cláudia Faria, Nádia Correia, Daniel Ribeiro, Alice Melão, Vanda Póvoa, Joana Matos, Joana Silva e Padma Akkapeddi. Um especial obrigado à Rita por todo o apoio disponibilizado durante a realização do projecto bem como durante a escrita desta tese. À Alice, obrigado por toda a ajuda na análise dos resultados, e por todas as conversas de incentivo sem as quais tudo teria sido mais complicado.

Gostaria, também, de agradecer à Ana Vieira e à Ana Sílvia Gonçalves da Unidade de Citometria de Fluxo do Instituto de Medicina Molecular pela preciosa ajuda técnica.

Gostaria de agradecer a contribuição dos doentes e respectivas famílias, e ao Director Clínico, Médicos, e Enfermeiros do Departamento de Pediatria do Instituto Português de Oncologia de Lisboa Francisco Gentil envolvidos na disponibilização de amostras primárias utilizadas neste projecto.

O meu sincero e grande obrigado aos meus pais e à minha irmã. Obrigado por me terem proporcionado a hipótese de frequentar o Mestrado em Oncobiologia, e obrigado por todo o apoio e carinho ao longo destes dois anos. Obrigado por estarem sempre disponíveis e com um sorriso para me dar, tanto nos bons como nos maus momentos.

Em último lugar, obrigado André por todo o amor e carinho demonstrados, mesmo quando o meu mau feitio tornava isso praticamente impossível.

Abstract

Acute lymphoblastic leukemia (ALL) is the most frequent childhood malignancy and it is characterized by the accumulation of immature lymphoid cells within the bone marrow and lymphoid tissues. Approximately 85% of pediatric ALL patients have a B-cell phenotype (B-ALL), and, despite significant improvements in treatment outcome, around 10-20% still relapse. Thus, there is a clear need for new prognostic factors capable of accurately predicting response to therapy.

PI3K/Akt/mTOR and JAK/STAT5 pathways are extensively implicated in cancer. Both cell-autonomous factors and microenvironmental cues, such as interleukin 7 (IL-7), contribute to the activation of these pathways in ALL. However, it remains to be determined whether their activation status has a prognostic value in this malignancy.

In the current thesis, we proposed to tackle this issue by analyzing the phosphorylation levels of key elements of both pathways in a retrospective cohort (n=58) of pediatric B-ALL cases. Methodologically, we decided to use phospho-flow cytometry, given its potential applicability in clinical diagnostics.

Overall, our results show that pediatric B-ALL samples display significant inter-patient heterogeneity in the constitutive and IL-7-triggered levels of PI3K/Akt/mTOR and JAK/STAT5 pathway activation. Interestingly, we found that the response to IL-7 does not correlate with the levels of IL-7 receptor α expression. Most importantly, correlation of basal activation levels of both pathways with clinical features with known prognostic value revealed that higher constitutive levels of phosphorylation of S6 on S235/236 and Akt on S473, but not on T308, are associated with higher white blood cell counts. These results suggest the existence of two independent mechanisms leading to Akt activation in ALL, with different biological outcomes.

Overall, our preliminary results suggest that there is a positive association of high Akt S473 and S6 S235/236 phosphorylation levels with high risk, which is often associated with a poor prognosis.

Keywords: B-cell Acute Lymphoblastic Leukemia, PI3K/Akt/mTOR pathway, JAK/STAT5 pathway, Interleukin 7, Prognostic value.

Resumo

A leucemia linfoblástica aguda (LLA) é o cancro mais frequente em crianças, apresentando um pico de incidência entre os 2 e os 5 anos de idade. Esta doença caracteriza-se pela expansão clonal descontrolada e consequente acumulação de linfócitos imaturos na medula óssea, com posterior infiltração de outros órgãos. O subtipo mais comum de LLA é a leucemia linfoblástica aguda de células B (LLA-B), constituindo cerca de 85% dos casos pediátricos e 75% dos casos adultos. Os tratamentos actuais apresentam uma elevada eficácia e aproximadamente 80% dos doentes pediátricos apresentam-se livres de doença 5 anos após o início do tratamento. Contudo, cerca de 10-20% dos doentes sofrem recidivas, frequentemente associadas a complicações a longo prazo, resultantes da elevada toxicidade dos tratamentos. Existem vários factores de prognóstico em LLA pediátrica essenciais para definir o tratamento mais adequado dos doentes, incluindo idade, contagem de leucócitos na fase de diagnóstico e presença de anomalias citogenéticas (trissomia 21 ou cromossoma de Filadélfia). Um factor de grande importância para a progressão da doença é a activação de vias de transdução de sinal fundamentais. Sabe-se, por exemplo, que mutações em elementos destas vias podem afectar a resposta dos doentes ao tratamento. No entanto, e apesar da contribuição destas vias para o desenvolvimento de LLA-B, o seu valor prognóstico não é conhecido. Importa salientar que a caracterização da activação das vias de transdução de sinal à data do diagnóstico poderá auxiliar na escolha de terapias mais específicas, com consequente aumento da eficácia e diminuição da toxicidade do tratamento.

As vias de sinalização PI3K/Akt/mTOR and JAK/STAT5 têm sido amplamente implicadas em cancro de um modo geral e, em particular, em LLA. A via PI3K/Akt/mTOR encontra-se constitutivamente hiperactivada em doentes pediátricos com leucemia linfoblástica aguda de células T (LLA-T), promovendo a viabilidade das células leucémicas. Foi também demonstrado que esta via é activada pela citocina IL-7 (que se encontra presente no microambiente tumoral), modulando a resistência das células leucémicas face à quimioterapia. A corroborar este facto, diferentes estudos indicam que a citocina IL-7 é capaz de modular, tanto *in vitro* como *in vivo*, a resposta das células de LLA-B a inibidores farmacológicos de mTOR (Rapamicina). Existe igualmente evidência a nível genético que apoia um possível valor prognóstico para esta via em LLA. Vários estudos realizados em LLA-T mostram que mutações ou delecções que levam à inactivação do principal regulador negativo da via, o supressor tumoral PTEN, estão associadas a um pior prognóstico. Este regulador pode ainda estar sujeito a inactivação pós-tradução, um processo bastante frequente tanto em LLA-T como em LLA-B.

Tal como a via PI3K/Akt/mTOR, a via de sinalização JAK/STAT5 é activada em resposta a estimulação com IL-7, ou quando o receptor desta citocina, IL-7R, se encontra constitutivamente activado devido a mutações. O principal papel desta via no desenvolvimento de LLA-B tem sido maioritariamente demonstrado pela activação constitutiva do factor de transcrição STAT5 a jusante da translocação BCR-ABL. Doentes com esta translocação, conhecidos como Filadélfia-positivos, apresentavam outrora muito mau prognóstico, uma situação resolvida com a inclusão no tratamento de terapias direccionadas especificamente para BCR-ABL, entre as quais o Imatinib foi o primeiro exemplo.

Tendo em conta as razões acima descritas, o principal objectivo desta tese é determinar, pela primeira vez, se o estado de activação das vias de sinalização PI3K/Akt/mTOR e JAK/STAT5 tem valor prognóstico em LLA-B pediátrica. Para responder a esta questão, avaliaram-se os níveis de fosforilação de elementos chave de cada uma das vias de transdução de sinal num grupo retrospectivo (n=58) de casos pediátricos de LLA-B provenientes do Departamento de Pediatria do Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG). Para determinar o estado de activação de PI3K/Akt/mTOR analisaram-se os níveis de fosforilação de Akt e de S6, um alvo de mTOR; quanto à segunda via, JAK/STAT5, avaliou-se o nível de fosforilação de STAT5. Estes níveis foram medidos tanto basalmente como após estimulação com IL-7, utilizando citometria de fluxo (*phospho-flow cytometry*). Posteriormente, e uma vez que dispomos dos dados clínicos de todos os doentes utilizados neste estudo, correlacionaram-se os valores de fosforilação obtidos com os parâmetros clínicos com valor prognóstico, tais como idade, contagem de leucócitos à data do diagnóstico e doença residual mínima após a terapia de indução. Correlacionaram-se, também, com o estado de maturação de LLA-B (classificação de *EGIL*), com o objectivo de melhor compreender a biologia da doença. Adicionalmente a esta análise molecular, procedeu-se a uma análise funcional onde se avaliou a sensibilidade de cada amostra primária à citocina IL-7, medindo parâmetros como a viabilidade e a proliferação das células primárias em resposta à IL-7. Mediram-se, ainda, os níveis de expressão da subunidade α do IL-7R (IL-7Rα) nestas amostras, com o intuito de os correlacionar tanto com os resultados moleculares como com os funcionais.

É importante referir que a metodologia *phospho-flow cytometry* foi seleccionada tendo por base a enorme quantidade de informação passível de ser obtida através da análise de uma única célula, e também por facilmente poder ser introduzida como técnica de diagnóstico em contexto clínico num futuro próximo. Na verdade, a técnica de citometria de fluxo já é actualmente utilizada na clínica para proceder à sub-classificação dos doentes com LLA com base no imunofenótipo das células.

Tendo em conta os nossos resultados, demonstrou-se que as amostras pediátricas de LLA-B são bastante heterogéneas no que diz respeito aos níveis de activação constitutiva das vias de transdução de sinal PI3K/Akt/mTOR e JAK/STAT5. Verificou-se, também, que a estimulação com IL-7 induz um aumento de activação de ambas as vias, embora com grande variabilidade entre as amostras. Quanto à análise funcional, e em concordância com o que já se tinha observado, a maioria das amostras primárias é sensível à estimulação com IL-7, traduzindo-se num aumento de viabilidade e proliferação celular. No que diz respeito aos níveis de expressão do IL-7Rα, também eles bastante variáveis, verificou-se que os mesmos não se correlacionam com os resultados moleculares e/ou funcionais. Isto é, níveis elevados de expressão do receptor não se traduzem necessariamente em maior activação das vias após estimulação com IL-7, nem num maior aumento de viabilidade ou proliferação celular. Para terminar, procedeu-se à correlação dos níveis de activação de ambas as vias de sinalização, tanto basais como após estimulação com IL-7, com as características clínicas anteriormente mencionadas. Não se encontrou nenhuma correlação significativa quando os níveis de activação foram comparados com a idade, o estado de maturação ou a doença residual mínima. Curiosamente, níveis basais elevados de fosforilação de S6 nas serinas 235 e 236 e de Akt na serina 473 (mas não na treonina 308) correlacionam-se com níveis elevados de leucócitos no diagnóstico que, por sua vez, estão associados a um risco elevado. Compararam-se, também, os níveis de expressão do IL-7Rα com os mesmos parâmetros clínicos e, embora não se tenha encontrado nenhuma associação significativa, existe uma tendência para níveis elevados de expressão em crianças com idade igual ou superior a 10 anos, normalmente associada a um pior prognóstico.

Concluindo, estes resultados, embora preliminares, parecem sugerir uma possível associação entre níveis elevados de fosforilação de S6 (serinas 235 e 236) e Akt (serina 473) e risco elevado, que se encontra normalmente associado a um mau prognóstico. O facto de esta correlação apenas abranger a fosforilação de Akt na serina 473, e não a na treonina 308, aponta para possível existência de dois mecanismos de activação de Akt em LLA, afectando diferencialmente os dois resíduos com consequências biológicas distintas. É nossa intenção repetir as análises realizadas neste estudo num maior número de amostras primárias, com o objectivo de validar as conclusões apresentadas nesta tese.

Palavras-chave: Leucemia linfoblástica aguda de células B (LLA-B), via de sinalização PI3K/Akt/mTOR, via de sinalização JAK/STAT5, citocina IL-7, valor de prognóstico.

Abbreviations

μHC	Cytoplasmic μ heavy chain
7-AAD	7-Aminoactinomycin D
β-ME	β-mercaptoethanol
ABL1	Abelson murine leukemia viral oncogene homolog 1
AEBSF	4-(2-Aminoethyl) enzenesulfonyl fluoride hydrochloride
Akt/PKB	v-akt murine thymoma viral oncogene homolog/Protein kinase B
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AnnV	Annexin V
APC	Allophycocyanin
B-ALL	B-cell Acute Lymphoblastic Leukemia
BAD	BCL2-associated agonist of cell death
Bcl-2	B-cell CLL/Lymphoma 2
Bcl-X_L	BCL2-like 1 isoform 1
BCR	B cell receptor
BCR	Breakpoint cluster region gene
BM	Bone marrow
cDNA	coding Deoxyribonucleic acid
CD	Cluster of differentiation
CNS	Central nervous system
CRLF2	Cytokine receptor-like factor 2
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
cyIg	Cytoplasmic immunoglobulin
DI	DNA Index
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D_H	Immunoglobulin heavy chain diversity region
E2A	Immunoglobulin enhancer-binding factor
EBF	Early B cell factor
EDTA	Ethylenediaminetetraacetic Acid
EGIL	European Group for the Immunological classification of Leukemias
ETV6	Ets variant 6

FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
Flt3	Fms-like tyrosine kinase 3
FSC-A	Forward scatter area
FSC-W	Forward scatter width
G-CSF	Granulocyte colony-stimulating factor
GL	Germline
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK3β	Glycogen synthase kinase 3β
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
iAMP21	Intrachromosomal amplification of chromosome 21
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL-7	Interleukin 7
IL-7R	IL-7 receptor
JAK	Janus kinase
J_H	Immunoglobulin heavy chain joining region
J_L	Immunoglobulin light chain joining region
Mcl-1	Induced myeloid leukemia cell differentiation protein
MFI	Mean fluorescence intensity
MLL	Mixed lineage leukemia
MRD	Minimal residual disease
mTOR	Mammalian target of rapamycin
Pax5	Paired box 5
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDKs	3-Phosphoinositide dependent protein kinases
PE	Phycoerythrin
Ph-ALL	Philadelphia-positive Acute Lymphoblastic Leukemia
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate

PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
Pre-B	Precursor B cells
Pro-B	Progenitor B cells
PTEN	Phosphatase and tensin homolog
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor
SCT	Stem cell transplantation
SDF-1	Stromal derived factor 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
sIg	Surface immunoglobulin
SSC-A	Side scatter area
STAT	Signal Transducer and Activator of Transcription
T-ALL	T-cell Acute Lymphoblastic Leukemia
TBS-T	Tris-Buffered Saline with Tween 20
TdT	Terminal deoxynucleotidyl transferase
TKIs	Tyrosine kinase inhibitors
TSLP	Thymic stromal lymphopoietin
V_H	Immunoglobulin heavy chain variable region
V_L	Immunoglobulin light chain variable region
VDJ	Variable-diversity-joining
WBC	White blood cell
XSCID	X-linked severe combined immunodeficiency

Contents

Index of Figures

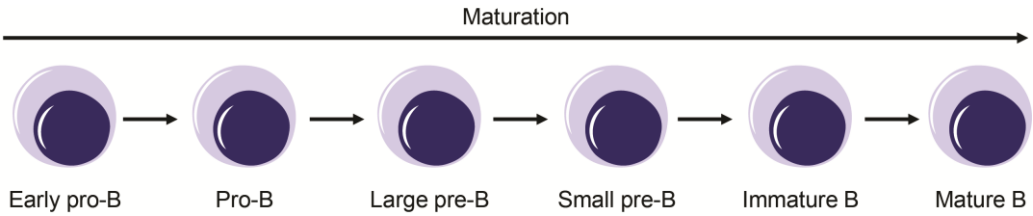
Index of Tables

Introduction

B cell development

The development of B lymphocytes is an ordered and highly regulated process that begins in the bone marrow (BM) and continues in the secondary lymphoid organs, such as the spleen and lymph nodes. It starts when hematopoietic stem cells (HSCs) become committed to the B cell lineage and proceeds with the rearrangement of immunoglobulin (Ig) genes, as well as with the gain and loss of expression of several genes that have critical roles in commitment and maintenance along the B cell development[1].

B lymphopoiesis can be divided into several stages, each defined by the sequential expression of different genes, and by the rearrangement status of the immunoglobulin heavy (IgH) and light (IgL) chains (Figure 1). The earliest cell committed to the B cell lineage is called the early progenitor-B cell (early pro-B or pre-pro-B), which is characterized by the beginning of IgH chain rearrangement, with the recombination of diversity (D_H) and joining (J_H) gene segments, and the expression of Igα (CD79a), a B lineage specific protein[2-4]. In pro-B cells, the rearrangement of the variable (V_H) gene segment with the rearranged DJ_H segments occurs and cells start to express CD19[2, 3, 5]. Upon completion of functional VDJ IgH rearrangements, pro-B cells differentiate into precursor B cells (pre-B cells). Pre-B cells are characterized by the expression of the cytoplasmic μ heavy chain (μHC) on the cell surface, in association with a surrogate light chain, as part of the pre-B cell receptor (pre-BCR) complex, which plays important roles in B cell proliferation and maturation[6, 7]. This stage can be subdivided into two substages: an initial proliferative phase called large pre-B cell stage and a maturation phase named small pre-B cell stage, which is characterized by the rearrangement of the V_L and J_L gene segments of the IgL chain[7]. Signaling through the pre-BCR is responsible for the transition between these two substages. After the successful IgL rearrangement and association with the HC, an IgM is produced, which specifically characterizes immature B cells. The immature B cells that are not self-reactive leave the BM and complete their maturation in the spleen.



	Early pro-B	Pro-B	Large pre-B	Small pre-B	Immature B	Mature B
CD34	+	+	-	-	-	-
CD10	+	+	+	+	+	-
CD19	-	+	+	+	+	+
TdT	-	+	-	-	-	-
CD79a	+	+	+	+	+	+
pre-BCR	-	-	+	-	-	-
IgH	DJ	VDJ	VDJ	VDJ	VDJ	VDJ
IgL	GL	GL	GL	VJ	VJ	VJ
IgM	-	-	-	-	+	+

Figure 1. Schematic representation of B cell developmental stages. Each stage is defined by the sequential expression of different proteins and by the rearrangement status of the IgH and IgL chains. GL: germline; TdT: terminal deoxynucleotidyl transferase.

As described earlier, B cell development is a tightly regulated process, mainly controlled at the transcriptional level. This level of regulation is responsible for B cell lineage commitment and differentiation. By knockout experiments, several transcription factors have been identified as essential for B lymphopoiesis. In lymphoid lineage commitment, two transcription factors are the key regulators, PU.1 and Ikaros[8, 9]. PU.1 is also responsible for controlling the expression of the interleukin 7 receptor (IL-7R) in lymphoid progenitors[10]. Other three transcription factors, immunoglobulin enhancer-binding factor (E2A), early B cell factor (EBF) and paired box protein (Pax5), are important for the commitment to the B cell lineage at the pro-B cell stage[11]. Although the transcription factors mentioned above affect early development, some of them are also active at the later stages of B cell differentiation.

Besides the importance of the specific transcription factors mentioned above, the cells comprising the BM microenvironment also have a major role in B cell development by directly interacting with the B cell precursors and by secreting required cytokines and chemokines. Thus, both intrinsic and extrinsic factors regulate B cell lymphopoiesis. Receptors to the secreted cytokines and chemokines are expressed in B cells and control the early stages of B cell development. The most important ones are c-kit, fms-like tyrosine kinase 3 (Flt3), IL-7R and C-X-C chemokine receptor type 4 (CXCR4). Their

respective ligands are the stem cell factor (SCF), Flt3 ligand (Flt3L), interleukin 7 (IL-7) and stromal derived factor 1 (SDF-1, also known as C-X-C chemokine ligand (CXCL) 12)[12, 13]. Different cell types within the BM, such as osteoblasts, reticular cells and fibroblast-like stromal cells expressing IL-7, produce these factors, creating specific cellular niches[12]. Throughout B cell development, B cells at different stages of differentiation localize within different BM niches according to their needs (Figure 2).

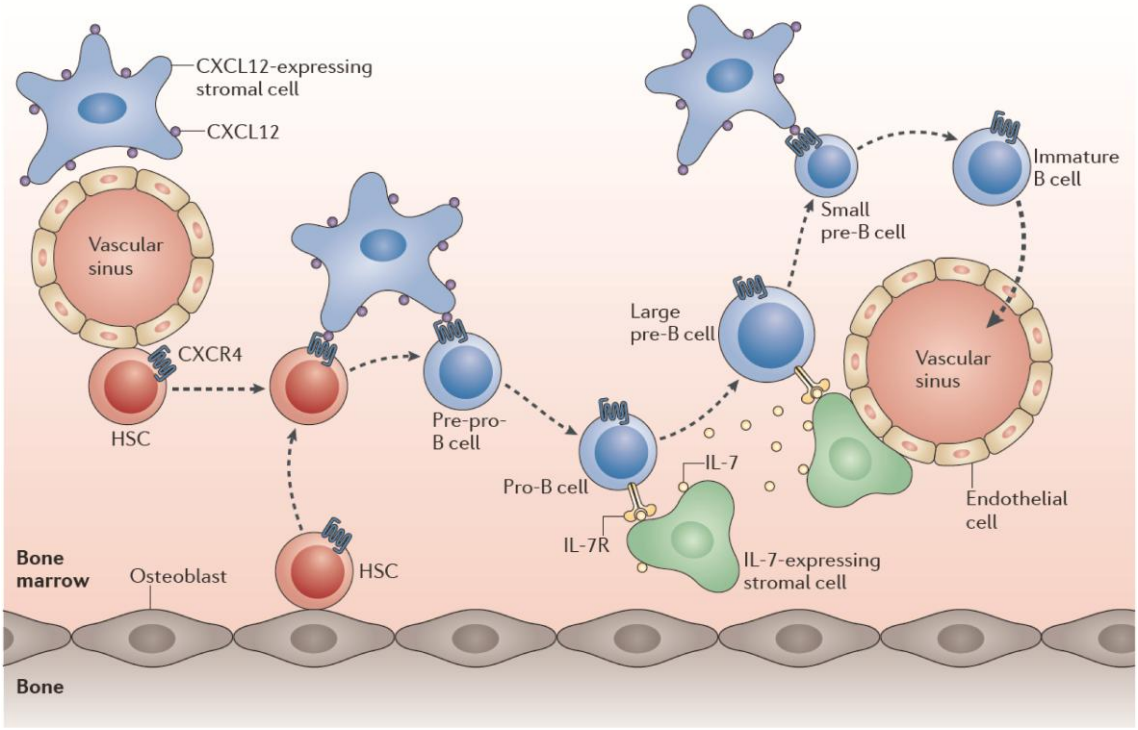


Figure 2. Movement of B cell progenitors within BM niches during B cell development. HSCs, when committed to the B cell lineage, move from the osteoblasts or endothelial cells to the reticular cells expressing CXCL12. Thereafter, pro-B cells move away from these cells towards IL-7-expressing stromal cells and, within the pre-B cell stage, large pre-B cells are still in contact with IL-7-expressing cells, whereas small pre-B cells leave them. At last, immature B cells exit the BM. From Clark *et al*., 2014[14].

B-cell Acute Lymphoblastic Leukemia (B-ALL)

Malignant B cell development

B-ALL is a malignant neoplasm characterized by uncontrolled clonal proliferation and accumulation of immature cells of B lymphoid lineage within the BM that ultimately leads to the infiltration of several extramedullary organs. These cells phenotypically resemble the normal developmental stages of B cell differentiation and can disrupt normal hematopoiesis due to their abnormal proliferative rate. The mechanisms that impair

normal B cell development and the precise cellular origin of the disease are not completely understood.

Regarding the mechanisms that lead to the disruption of B cell lymphopoiesis, it is already known that multiple specific genetic changes may contribute to B-ALL development. It is thought that a primary genetic event, usually a chromosome translocation, followed by different secondary genetic alterations may drive the malignant transformation. These genetic alterations commonly interfere with the control of normal B cell differentiation and proliferation[15]. In fact, alterations in genes encoding transcription factors essential for normal B lymphopoiesis, such as Ikaros, E2A, EBF and Pax5, have been described[16]. As in normal development, genetic abnormalities are not the only events promoting leukemogenesis: the surrounding microenvironment plays an important role in this process. Indeed, both composition and function of BM stromal cells are significantly changed in B-ALL cases, leading to an increase in the levels of the chemokines and cytokines responsible for the proliferation and survival of the leukemic cells[17]. However, it is not known whether the microenvironmental abnormalities precede the leukemic stage or are a consequence of the leukemic cell activity.

The exact cell-of-origin of B-ALL remains an open relevant issue. By *in vitro* and *in vivo* studies, it has been suggested that the primary genetic event can occur in different cells at different stages of maturation[18]. In other words, both cells at early stages of B cell development as well as cells at later stages can be the leukemia cells-of-origin in human B-ALL. Another hypothesis defends that the primary transformation may take place at an early developmental stage, followed by further differentiation of the altered B cell and arrestment at a later stage of differentiation[19]. Regarding pediatric B-ALL, it has been proposed, for several years, that it may be originated prenatally *in utero* during fetal hematopoiesis, with additional postnatal events to complete malignant transformation[20].

General Features

Acute lymphoblastic leukemia (ALL) is the most frequent childhood malignancy, accounting roughly for one quarter of all pediatric cancers and more than 80% of leukemias. ALL has a peak of incidence at 2 to 5 years of age and is also common in the elderly, while it is unusual in middle-aged adults[21, 22]. The most common subtype of ALL is B-ALL, comprising 85-90% of pediatric and 75% of adult ALL cases. Despite significant improvements in treatment outcome, with a 5-year event-free survival rate of approximately 80% for childhood ALL[23], around 10-20% of the patients still relapse with very poor prognosis. The scenario is even worst when looking at adult ALL, where only

about 30-40% of the patients achieve long-term disease-free survival[24]. Of note, the current thesis focuses only on pediatric B-ALL.

Patients with ALL are usually characterized by BM infiltration with more than 80-100% of malignant lymphoblasts and diagnostic symptoms are correlated with leukemia cell burden, extension of BM replacement and involvement of peripheral blood (PB) and extramedullary sites. For these reasons, there are several clinical features at presentation, such as anemia, thrombocytopenia, splenomegaly, lymphadenopathy and headache (usually an indication of central nervous system (CNS) involvement)[25].

B-ALL can be sub-classified according to the immunophenotype and to specific genetic abnormalities. Regarding the immunophenotype, the European Group for the Immunological classification of Leukemias (EGIL)[26] proposes a classification mainly based on the maturation stage of normal B cell development where the arrest occurred, leading to four B-ALL subtypes: pro-B ALL (B-I), common B-ALL (B-II), pre-B ALL (B-III) and mature B-ALL (B-IV, Burkitt ALL). The first three subtypes constitute the precursor B-ALL group, whereas the last one forms the mature B-ALL group. The most common subtype in pediatric cases is, as the name suggests, the common B-ALL. A summary of the immunological markers of each subtype is shown in Table 1.

Table 1. The EGIL classification of pediatric B-ALL.

B-ALL Subtype	Immunological markers
Pro-B ALL	HLA-DR$^+$, TdT$^+$, cCD79$^+$, CD19$^+$, CD10$^-$, cyIgM$^-$, CD20$^-$, sIg$^-$
Common B-ALL	HLA-DR$^+$, TdT$^+$, cCD79$^+$, CD19$^+$, CD10$^+$, cyIgM$^-$, CD20$^-$, sIg$^-$
Pre-B ALL	HLA-DR$^+$, TdT$^+$, cCD79$^+$, CD19$^+$, CD10$^+$, cyIgM$^+$, CD20$^{+/-}$, sIg$^-$
Mature B-ALL	CD20$^+$, sIg$^+$, TdT$^-$

HLA: human leukocyte antigen; TdT: terminal deoxynucleotidyl transferase; cy: cytoplasmic; s: surface.

Regarding the genetic abnormalities, they are detected in about 80% of children with B-ALL and their identification has important implications on prognosis and therapeutic choices[15]. Different types of alterations have been found, including chromosomal translocations; additions or deletions of chromosomes and genes; and rearrangement, gain, loss or mutation of specific genes. These genetic changes often lead to disruption of the regulation of normal B cell development and aberrant signal transduction, by affecting the function of transcription factors and the constitutive activation of tyrosine kinases,

respectively[15]. The frequency of the genetic abnormalities seen in pediatric cases is presented in Figure 3.

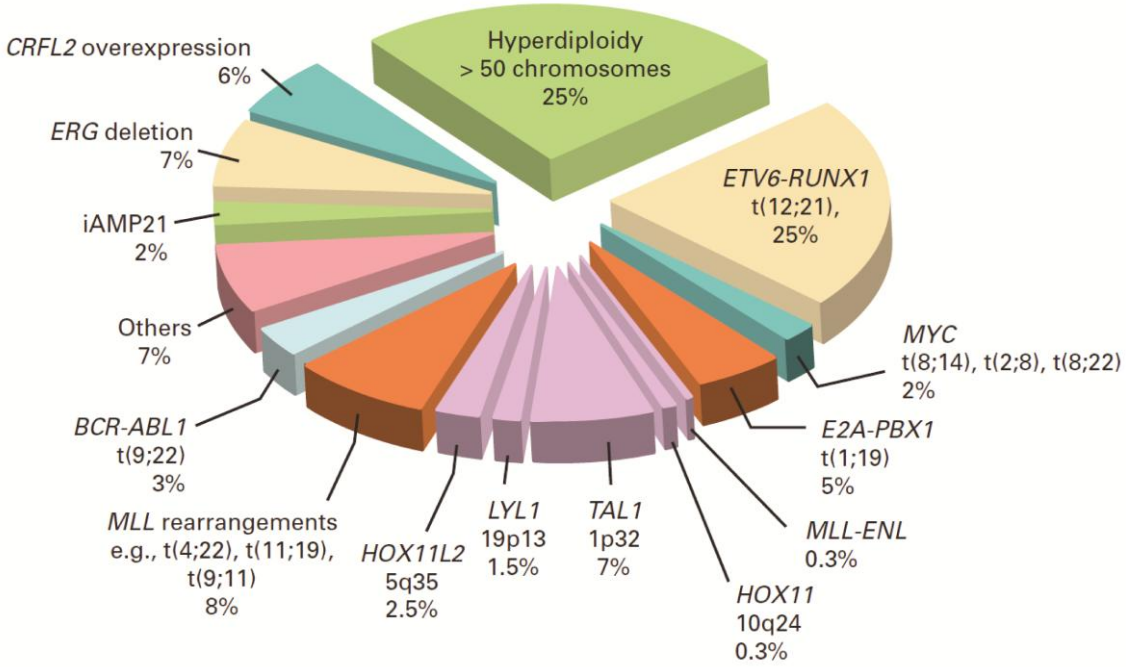


Figure 3. Estimated frequency of genetic abnormalities subgroups in pediatric ALL. The genetic alterations shown in purple are only detected in T-cell ALL cases. This figure does not include submicroscopic genetic alterations. iAMP21: intrachromosomal amplification of chromosome 21. Adapted from Pui *et al*., 2011[23].

Treatment

Childhood ALL treatment has been changing over time in order to establish taylor-made, patient-adjusted therapies with the goal of augmenting efficacy while decreasing the significant acute toxicities and late-occurring adverse events associated with current chemotherapy.

Briefly, B-ALL treatment usually comprises 3 phases. The initial phase of treatment, the remission induction phase, whose main objective is to achieve remission and restore normal hematopoiesis, includes the combination of glucocorticoids (prednisone or dexamethasone), vincristine and asparaginase. One or more additional drugs, such as an anthracycline (doxorubicin or daunorubicin) and cyclophosphamide, are integrated in the treatment of children with high or very high risk ALL[27]. After completion of this first phase, it is necessary to ensure the complete abolishment of residual leukemic cells, including the drug-resistant ones. This is the goal of the consolidation or

intensification phase, normally consisting on the administration of high-dose methotrexate and mercaptopurine[27]. The treatment finishes with a less intensive regimen of weekly methotrexate and daily mercaptopurine, named continuation or maintenance therapy, to avoid relapses and effect cure[27]. Since some patients may present CNS involvement at time of diagnosis and some of them may present it during the course of the disease, CNS directed therapy should be included in treatment protocols. Currently, this therapy consists of triple intrathecal chemotherapy with methotrexate, cytarabine, and hydrocortisone[27].

It is important to refer that pediatric patients with mature B-ALL, known to have unfavorable prognosis, are treated with a more intensive chemotherapy regimen, including high-dose methotrexate, cytarabine, and cyclophosphamide, for a short period of time[21]. It has also been shown that high risk ALL patients, such as those with Ph-positive disease and those with a poor early response to treatment, benefit from allogeneic hematopoietic stem cell transplantation (SCT), the most intensive form of treatment[21].

Prognostic Factors

The prognosis of childhood ALL has improved substantially over the last decades, mostly due to the identification of trustworthy prognostic factors essential to establish prognosis, treatment protocol and follow-up strategy, as well as to personalize patient counseling. Two subgroups of prognostic factors can be identified: those at presentation, including age, initial white blood cell (WBC) counts, CNS disease, immunophenotype and presence of certain cytogenetic and molecular features; and those based on treatment response, comprising achievement of complete remission, detection of minimal residual disease (MRD), time to relapse and overall survival[23]. Notably, some prognostic indicators in pediatric ALL, such as gender and race, have lost their prognosis strength as a result of enhanced treatment strategies[21].

Regarding the first subgroup of prognostic factors, and starting by age at diagnosis which has a strong prognostic effect, children older than 1 year of age and younger than 10 years of age have a better prognosis than infants (less than 1 year old) or adolescents. In fact, the 5-year event-free survival estimate is 88% for children with ages between 1 and 9 years, 73% for adolescents between 10 and 15 years, 69% for children older than 15 years, and 44% for infants. The latter group has the worst prognosis[21]. Other factors conferring a poor prognosis are the presence of high WBC counts ($\geq 50 \times 10^9$cells/L) and CNS disease at diagnosis[21]. The prognostic value of the developmental stage of B-ALL is uncertain. It is currently known that children with mature B-ALL have an unfavorable prognosis as compared to those with precursor B-ALL[21]. Nonetheless, there is no

consensus about the prognostic power of each of the three subtypes within the precursor B-ALL group. The last, but not least, important prognostic factor in this subgroup is the presence of genomic abnormalities. The most commonly associated with an adverse outcome are hypodiploidy (<44 chromosomes), especially near haploidy (24-31 chromosomes) and low-hypodiploidy (32-39 chromosomes); *BCR-ABL1* translocation (also called Philadelphia chromosome (Ph)); *MLL* gene rearrangements; and iAMP21[28]. Of note, the outcome of patients with *BCR-ABL1* translocation has been dramatically improved with the introduction of BCR-ABL targeted therapies (tyrosine kinase inhibitors, TKIs, such as Imatinib) in the treatment protocols. Those associated with a favorable prognosis are hyperdiploidy (>50 chromosomes) or DNA index (DI) ≥1.16, and *ETV6-RUNX1* rearrangement[28]. This is confirmed by the estimated event-free survival rates for each genetic abnormality (Table 2)[27].

Table 2. Estimated event-free survival of different genetic abnormalities with significant prognostic value in pediatric B-ALL.

Prognosis	Genetic abnormality	Estimated percent event-free survival (at the indicated years)
Favorable	Hyperdiploidy	80-90 (5 years)
	ETV6-RUNX1 fusion	85-95 (5 years)
Adverse	Hypodiploidy	30-40 (3 years)
	BCR-ABL1 fusion	80-90 (3 years)
	MLL-AF4 fusion	30-40 (5 years)
	iAMP21	60-70 (5 years)

Regarding the response to treatment, MRD detection at the end of induction therapy is the most powerful independent predictor of prognosis and can be used to change treatment regimens[28, 29]. Assessment of this parameter provides a more sensitive measurement of the rate of reduction of leukemic cells from both BM and PB during remission induction therapy[28]. Two different methods can be used to detect MRD at very low levels (<0.01%), namely flow cytometric profiling of aberrant leukemia-associated immunophenotype and polymerase chain reaction (PCR) amplification of leukemia-specific fusion transcripts or *Ig* gene rearrangements[21, 25]. Children with 1% or more leukemic cells in BM at the end of remission induction therapy have a worse prognosis, whereas those with less than 0.01% leukemic cells, which achieved immunological or molecular remission, have an outstanding outcome[21]. So, the extent of MRD inversely correlates with prognosis.

IL-7/IL-7R-mediated signaling in normal and malignant B cell development

Normal B- and T- cell development require different cytokines secreted in the microenvironment, including IL-7, a type I pro-survival cytokine originally described as a growth factor for mouse B lineage progenitors[30]. IL-7 is produced by several types of cells, such as BM and thymic stromal cells, vascular endothelial cells, intestinal epithelium, keratinocytes and follicular dendritic cells. Its heterodimeric receptor, IL-7R, comprises the IL-7Rα subunit (CD127), encoded by *IL7R* and shared by the receptor for thymic stromal lymphopoietin (TSLP), and the common gamma chain, γc, which is shared by the cytokine receptors for IL-2, IL-4, IL-9, IL-15, and IL-21[31, 32]. The IL-7 receptor is mainly expressed by lymphoid cells, as B and T cell precursors, and also by leukemic cells[33].

The precise role of the IL-7/IL-7R system during normal human B cell development is not as clear as it is for normal human T cell development and murine B cell development. Expression of the IL-7R is tightly regulated during both mouse and human B cell differentiation, starting at early pro-B cells until the large pre-B cell stage. When the maturation proceeds, its expression begins to be downregulated[33, 34]. The critical roles of IL-7 in murine B cell development have been demonstrated by *in vivo* loss-of-function experiments. By using blocking antibodies against IL-7 or IL-7R, it was observed a rapid decrease in the total number of B lineage cells within the BM[35, 36]. Furthermore, targeted deletions in the *IL-7*, *IL-7Rα* or *γc* genes lead to a block at an early stage of B lymphopoiesis in adult mice[37-39]. However, when one looks at human B cell development, IL-7 does not seem to be absolutely essential. Indeed, B cell differentiation can occur in the absence of IL-7, as it was shown by Pribyl and LeBien[40]. Using *in vitro* cultures of $CD34^{++}/CD19^{-}$ HSCs isolated from human fetal BM, the authors generated $CD19^{+}$ B cells without addition of exogenous IL-7. Moreover, addition of anti-IL-7 neutralizing antibodies to those cultures did not affect the development and expansion of $CD19^{+}$ cells[40]. This lack of IL-7 requirement is further supported by patients with X-linked severe combined immunodeficiency (XSCID), characterized by mutations in the γc subunit of the IL-7R, who completely lose T cells without affecting B cell numbers[41]. In spite of this apparent human IL-7-independent development of B cells, this cytokine still plays a role in cell survival, proliferation and differentiation during the early stages of B cell development[42].

Additionally to its role in normal lymphoid development, IL-7 has also been associated with ALL development. Indeed, IL-7 is present in the leukemia microenvironment[43] and leukemic cells express the IL-7R[33]. Several studies have addressed this issue, although the majority of them focused on T-cell acute lymphoblastic leukemia (T-ALL), the less common subtype of ALL. For instance, *in vitro* studies indicate

that IL-7 increases viability and promotes cell proliferation of primary T-ALL cells, acting mainly through activation of the PI3K/Akt pathway[31, 44, 45]. In addition, IL-7 was shown to accelerate leukemia expansion in murine xenotransplant models of T-ALL[46]. Regarding B-ALL development, although some studies have demonstrated that IL-7 induces proliferation and survival of primary B-ALL cells, the response to IL-7 varies considerably among patients[47, 48]. There is also evidence that IL-7R expression is elevated in adult patients with pre-B ALL[49]. Recently, somatic gain-of-function mutations in *IL7R* in pediatric B-ALL patients have been described, hinting on the oncogenic potential of IL-7R-mediated signaling in B cells[50]. It should be noted, nonetheless, that mutational activation of the IL-7R was frequently concomitant with aberrant expression of CRLF2, which together form a functional receptor for TSLP[50].

To exert its functions in cell survival and proliferation, IL-7 activates two important signaling pathways, JAK/STAT5 and PI3K/Akt/mTOR[31] (Figure 4). Activation of these key pathways has been extensively implicated in cancer in general and in ALL progression in particular. Importantly, mutations in elements of these pathways may impact on response to treatment[51-53]. The signaling is initiated by binding of the IL-7 to its receptor, which induces dimerization of the IL-7R. This, in turn, brings the associated Janus kinases (JAKs) proteins (JAK1 in IL-7Rα and JAK3 in γc) together and leads to their transphosphorylation and activation[54, 55]. Activated JAKs are then responsible for the phosphorylation of specific tyrosine residues on the cytoplasmic domain of the IL-7Rα subunit, thus creating docking sites for SH2-containing proteins, such as signal transducer and activator of transcription (STAT) proteins and phosphatidylinositol 3-kinase (PI3K)[54].

JAK/STAT5 pathway

Once STAT5 (two isoforms: STAT5a and STAT5b) is recruited and activated by JAK-mediated phosphorylation, it dimerizes and translocates to the nucleus, where it acts as a transcription factor of several genes whose proteins are involved in cell viability, proliferation and cell cycle progression[54]. Such proteins include Bcl-2, Bcl-X_L, Mcl1 and cyclin D3. Besides being activated in response to IL-7 stimulation[44, 48], STAT5 can also be activated upon mutational activation of the IL-7R[50, 56]. STAT5 is a crucial effector of IL-7 signaling during early B cell development, as demonstrated by the fact that expression of a constitutively active form of STAT5b (*Stat5b-CA*) largely restores B cell development in *Il7r*$^{-/-}$ mice[57]. There is evidence that STAT5 is involved in IL-7-mediated cell survival of pro-B cells by promoting the transcription of the *Mcl1* gene[58]. However, its involvement in regulating B cell differentiation is still controversial[59]. The key role of JAK/STAT5 pathway

in the development of human B-ALL is mainly shown by constitutive activation of STAT5 in Ph-positive ALL cases, downstream of the BCR-ABL translocation, and in patients with CRLF2 overexpression in combination or not with activating mutations in JAK1 and JAK2[59-61]. Mice with expression of a BCR-ABL cDNA in B cell progenitors developed a disease that resembles human B-ALL only in the presence of STAT5[62], further supporting the major contribution of this transcription factor in malignant transformation.

PI3K/Akt/mTOR pathway

Another signaling pathway activated downstream of IL-7/IL-7R axis is the PI3K/Akt/mTOR pathway. When activated by binding of the IL-7 to its receptor, PI3K, composed by a p110 catalytic subunit and a p85 regulatory subunit, is involved in the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) at the membrane[31, 63]. This event recruits and triggers the activity of the serine/threonine 3-phosphoinositide dependent protein kinases, PDK1 and PDK2, which are responsible for the phosphorylation and full activation of the serine/threonine kinase Akt/PKB. PDK1 phosphorylates a threonine residue (T308) within the kinase domain of Akt, whereas PDK2 phosphorylates a serine residue (S473) in the C-terminal domain of Akt[31, 63]. Once activated, Akt activates or represses by phosphorylation a number of downstream targets, such as BAD, GSK3β and mTOR[31]. mTOR phosphorylates and thereby activates p70S6 kinase, which then phosphorylates the ribosomal protein S6, leading to an increase in protein translation at the ribosome. Overall, this pathway promotes cell viability and proliferation, inhibits apoptosis and controls cell metabolism[31, 43, 63]. The major negative regulator of PI3K/Akt/mTOR pathway is the tumor suppressor PTEN, a lipid phosphatase that dephosphorylates PIP_3 into PIP_2, thus downregulating PI3K signaling[31, 63].

In vivo studies have shown that PI3K/Akt/mTOR pathway regulates cell proliferation of pre-B and also mature B cells, since mice deficient for the catalytic or regulatory subunits of PI3K have a block at the pre-B cell stage, with pro-B cells proliferating normally[64, 65]. However, it is not completely excluded a putative role of this pathway in the survival of pro-B cells in response to IL-7 signaling, acting in concert with STAT5[14]. Like the STAT5 pathway, the PI3K/Akt/mTOR pathway has also been associated with ALL. For instance, constitutive hyperactivation of PI3K/Akt/mTOR is a very frequent event in pediatric patients with T-ALL, involved in promoting leukemic cell viability[66]. Also, this pathway is activated by IL-7 present in the leukemia microenvironment, which may modulate resistance to chemotherapy[31, 67]. Indeed, some studies indicate that IL-7 can modulate the response of leukemic cells to mTOR

pharmacological inhibitors (Rapamycin) both *in vitro* and *in vivo*[68, 69]. It was also shown that mutational inactivation or deletion of the *PTEN* gene is associated with poor prognosis of ALL, raising the possibility that this pathway may have prognostic value in ALL[51, 52]. However, there is also evidence that non-deletional PTEN posttranslational inactivation occurs very often in both T-ALL[66] and B-ALL[70], which may be a confounding factor in prognosis analysis based exclusively on the mutational status of PTEN.

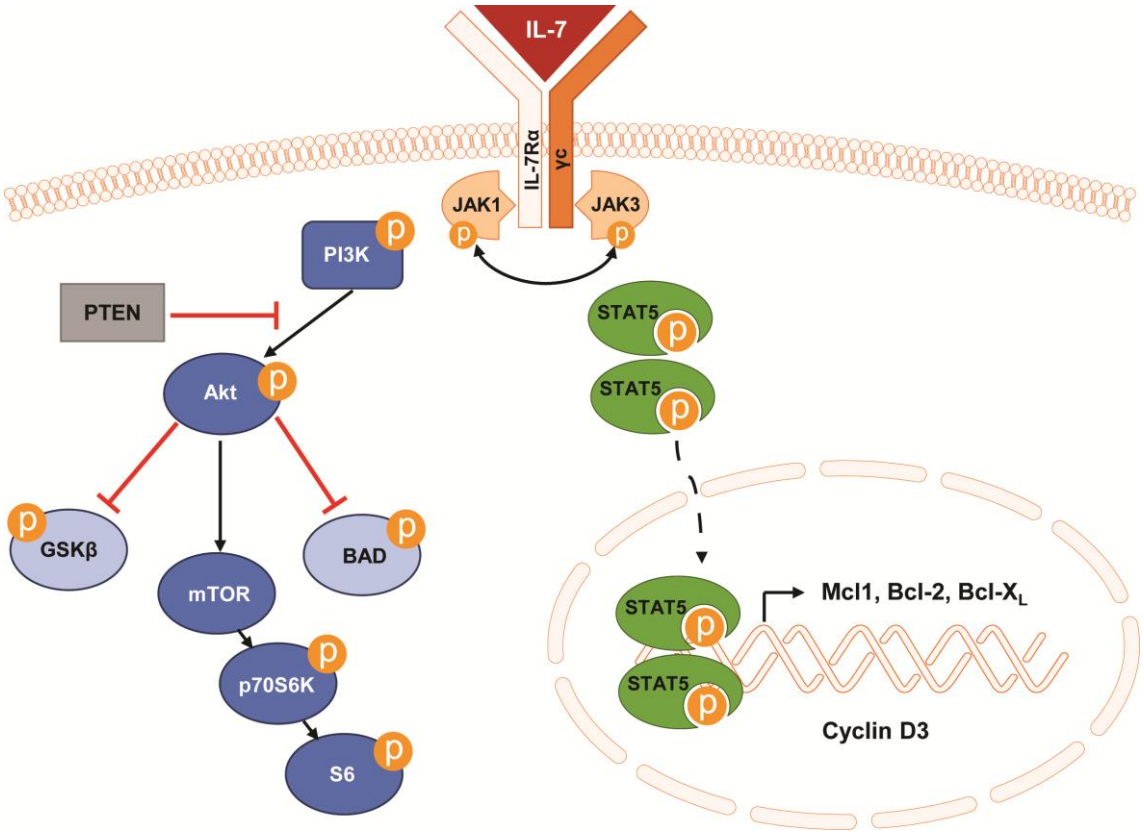


Figure 4. Schematic representation of IL-7/IL-7R-mediated signaling through JAK/STAT5 and PI3K/Akt/mTOR pathways. Black arrows denote activation, whereas red bars indicate inhibitory actions. Dashed arrow identifies translocation to the nucleus.

Objectives

Despite the significant improvements in treatment outcome over the last decades, around 10-20% of B-ALL patients still relapse and have very poor prognosis. So, there is an urgent demand for new prognostic factors predicting response to therapy. Given the importance of the PI3K/Akt/mTOR and JAK/STAT5 signaling pathways to B-ALL development, we decided to focus on these two critical pathways and evaluate the prognostic significance of their activation in B-ALL, at the protein level. Taken these reasons together, in the present thesis, we aimed to determine, for the first time, the levels of activation of PI3K/Akt/mTOR and JAK/STAT5 pathways in primary leukemia samples at the single cell level and correlate those levels with clinical parameters (such as MRD), in order to understand whether the activation status of these important signaling pathways have prognostic value in childhood B-ALL. To this purpose, we have used a flow cytometry approach with phospho-specific antibodies (phospho-flow) to analyze a retrospective cohort of pediatric B-ALL patient samples collected at diagnosis and for which we have collected clinical data. The reason why we selected the phospho-flow methodology relates to its potential applicability to actual clinical diagnostics.

The following specific aims were defined to accomplish the main goal:

1. Examine the basal levels of PI3K/Akt/mTOR and JAK/STAT5 signaling pathway activation for each patient sample.
2. Assess the levels of PI3K/Akt/mTOR and JAK/STAT5 activation for each patient sample upon *ex vivo* stimulation with IL-7.
3. Evaluate the sensitivity of the patient samples to IL-7.
4. Determine whether basal and/or IL-7-mediated activation of PI3K/Akt/mTOR and/or JAK/STAT5 signaling pathways may serve as prognostic factors.

In the first two specific aims, we have analyzed the phosphorylation levels of Akt, the mTOR downstream target S6 and STAT5.

By addressing these tasks, it was our overall goal to understand whether the activation status of PI3K/Akt/mTOR and/or JAK/STAT5 pathways has prognostic value in pediatric B-ALL.

Materials and Methods

Primary B-ALL samples and the NALM-6 cell line

Bone marrow samples from childhood B-ALL patients (n=58) were collected at diagnosis at the Pediatric Department of Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG) in accordance with the Declaration of Helsinki, after informed consent and proper internal Ethical Committee approval. Patient characteristics are summarized in Table S1 (see Supplementary Information). These samples were previously enriched by density centrifugation over Ficoll-Paque (GE Healthcare) and frozen in liquid nitrogen in a suspension of fetal bovine serum (FBS; Biowest) with 10% dimethyl sulfoxide (DMSO; Sigma). The human B-ALL cell line, NALM-6, was thawed and expanded in culture in RPMI-1640 culture medium with L-glutamine (Gibco) supplemented with 10% of FBS and penicillin/streptomycin (Gibco) (hereafter referred to as RPMI 10 medium) at a concentration of $0.5x10^6$ cells/ml at 37ºC in 5% CO_2. After three passages, this cell line was frozen in the same conditions described above.

Intracellular phospho-specific flow cytometry

To assess the phosphorylation status of PI3K/Akt/mTOR and JAK/STAT5 pathways, primary leukemia and NALM-6 cells were thawed and cultured in RPMI 10 at 37ºC in 5% CO_2 for 1 hour at a concentration of $2x10^6$ cells/ml and $0.5x10^6$ cells/ml, respectively. Afterwards, cells were washed with PBS (Gibco), pelleted by centrifugation, and fixed with Cytofix buffer (BD Biosciences) for 10 minutes at 37ºC. Cells were then pelleted and permeabilized in ice-cold Perm buffer III (BD Biosciences) for 30 minutes on ice. The cells were first washed in PBS and then in Stain buffer (BD Biosciences), and stained with the following antibodies: CD79a-APC (BioLegend); CD3-eFluor 450 (eBioscience); pAkt S473-Alexa Fluor 488, pAkt T308-PE, pS6 S235/236-Alexa Fluor 488, and pSTAT5 Y694-Alexa Fluor 488 (all from BD Biosciences). Following a 30 minutes incubation at room temperature in the dark, cells were washed in stain buffer, resuspended in PBS and analyzed on an LSRFortessa (BD Biosciences). To measure the phosphorylation status of both pathways upon *ex vivo* stimulation with IL-7 (Prepotech), the same protocol was performed with an additional step: before fixing the cells, they were stimulated with 50ng/ml of IL-7 and incubated at 37ºC for 30 minutes. At least 100 000 events were collected for all samples. Data were collected using DIVA software (BD Bioscience) and analyzed using the FlowJo software (Tree Star). Basal phospho-protein levels were normalized to those of NALM-6 cells, which underwent the same protocol.

Analysis of IL-7Rα surface expression

The expression of the interleukin 7 receptor α chain (IL-7Rα or CD127) on primary ALL cells was assessed *ex vivo* by flow cytometry using a CD127-PE antibody (eBioscience). For the staining, cells were washed with PBS and resuspended in PBS with CD19-APC (eBioscience), CD3-APC-eFluor 780 (eBioscience) and CD127-PE. After 30 minutes of incubation at 4ºC in the dark, cells were washed with PBS and resuspended in 200µL of PBS. Next, samples were analyzed in an LSRFortessa cytometer. Data were collected using DIVA software, analyzed with FlowJo software and represented as the specific mean fluorescence intensity (MFI).

Assessment of cell viability and cell size

Cells were cultured in 96-well plates at a density of $2x10^6$ cells/ml at 37ºC with 5% CO_2 in medium only or with 10ng/ml of IL-7. At different time points (24h, 48h, 72h or 96h), cells were harvested and cell viability was determined by double-staining with APC-conjugated Annexin V (AnnV; eBioscience) and 7-AAD (BD Biosciences). Briefly, cells were washed with PBS and resuspended in 100µL of PBS with FITC-conjugated CD19 (eBioscience) and CD3-eFluor 450. After 30 minutes of incubation at 4ºC in the dark, cells were washed with PBS and resuspended in 100µL of binding buffer (eBioscience) with Annexin V and 7-AAD. After 15 minutes of incubation at room temperature in the dark, 100µL of binding buffer were added and the samples analyzed by flow cytometry using an LSRFortessa. Live cells were identified as the Annexin V and 7-AAD double-negative population and cell size was evaluated by FSCxSSC discrimination within the live cell population. Data were collected using DIVA software and analyzed with FlowJo software.

Protein Extraction and Quantification

Cells were harvested and centrifuged at 3200 rpm for 5 minutes at 4ºC to produce a cell pellet. After discarding the supernatant, the cell pellet was lysed in lysis buffer (50mM Tris-HCl pH 8.0; 150mM NaCl; 5mM EDTA; 1% (v/v) NP-40, 1mM Na_3VO_4; 10mM NaF; 10mM NaPyrophosphate; supplemented with protease inhibitor cocktail Complete Mini (Roche)), supplemented with 1mM of AEBSF (Bio-Rad). Next, protein supernatants were collected by centrifugation at 13000 rpm for 20 minutes at 4ºC. The total protein was quantified by performing the Bradford assay (Bio-Rad). Before resolving the protein extracts, they were resuspended in Laemmli sample buffer (Bio-Rad) and denatured for 5 minutes at 95ºC.

Western Blot

Equal amounts of protein extracts were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes by using a conventional transfer system (90 minutes, 400mA). Then, in order to check the effectiveness and uniformity of the protein transfer, membranes were stained with Ponceau S solution (Sigma). Subsequently, membranes were blocked for 1 hour with 3% milk diluted in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T buffer) and immunoblotted with the following primary antibodies (previously diluted in TBS-T buffer): p-Akt (S473) (1:500) and Akt (1:1000) (Cell Signaling Technology); p-S6 (S235/236) (1:1000) and S6 (1:1000) (Cell Signaling Technology); p-STAT5A/B (Y694/Y699) (1:1000, Cell Signaling Technology) and STAT5 (1:1000, Santa Cruz Biotechnology); and actin (1:1000, Santa Cruz Biotechnology). The membranes were incubated with each antibody overnight at 4ºC under gentle agitation. As a reference, Precision Plus Protein™ molecular weight marker was used (Bio-Rad). Next day, the membranes were washed with TBS-T buffer for 25 minutes and incubated for 1 hour at room temperature under gentle agitation with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG (1:5000, Promega) or anti-goat IgG (1:5000, Santa Cruz Biotechnology), depending on the primary antibodies), diluted in 3% milk TBS-T buffer solution. After washing the membranes with TBS-T buffer for 25 minutes, immunodetection was performed by chemiluminescence detection using the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc.). Films exposed to the membranes were developed in a Curix60 (AGFA HealthCare).

Membrane Stripping

To re-probe the same membranes with new antibodies, the previous were removed through a stripping procedure. First, membranes were incubated for 30 minutes at 56ºC with slight agitation in stripping buffer (35mM Tris-HCl, 2% SDS, 1.42mM β-mercaptoethanol (β-ME), pH 6.7) supplemented with 14.2mM of β-ME (Bio-Rad). Afterwards, membranes were washed four times, being the first wash with water and the following ones with TBS-T buffer. At the end of the stripping procedure, the membranes were ready for a new immunoblot, starting from the blocking step of the protocol described above.

Proliferation Assays

Cells were cultured in flat-bottom 96-well plates at $2x10^6$ cells/ml at 37ºC with 5% CO_2 in RPMI 10 medium only or in the presence of 10ng/ml of IL-7. Cultures were performed in triplicates for the indicated time points. To assess DNA synthesis, cells were incubated with 1µCi/well of [^{3}H] thymidine (Perkin Elmer) for 16 hours before harvesting. [^{3}H] thymidine incorporation was measured by using a liquid scintillation counter (Perkin Elmer). Average and standard deviation of triplicates were calculated.

Statistical analysis

GraphPad Prism version 6.01 for windows (GraphPad Software) was used to perform statistical analysis. Differences between groups were calculated using Student's t test or One-way ANOVA, as appropriate. *P* values lower than 0.05 were considered statistically significant.

Results

Diagnostic ALL samples display heterogeneous levels of basal PI3K/Akt/mTOR and JAK/STAT5 signaling pathway activation

An important association between the activation status of oncogenic signaling pathways and their capacity to predict response to therapy and risk of relapse in acute myeloid leukemia (AML) patients was demonstrated by Gary Nolan and his colleagues[71]. Given this evidence, we decided to start by analyzing the basal levels of activation of the PI3K/Akt/mTOR and JAK/STAT5 pathways *ex vivo* by flow cytometry for each patient sample (Table S1). Using phospho-specific antibodies and focusing only on B-ALL blast cells, identified by the expression of cCD79a (Figure 5A), we assessed the levels of phosphorylation of Akt (at serine 473 and threonine 308), the mTOR downstream target S6 (at serines 235 and 236) and STAT5 (at tyrosine 694) (Figure 5B). This methodology was first established by Nolan *et al.*[71], and has been used and optimized in our lab[70]. In order to compare the patients between them, the basal levels were normalized to those of the B-ALL cell line, NALM-6, used in this study as a reference. This reference was included in every experiment. Briefly, each phospho-protein level was first normalized to the unstained condition and, then, to the respective phospho-protein level of NALM-6, previously normalized to the respective unstained condition as well. Overall, we found a high degree of variability between patients regarding the basal phosphorylation levels of each protein analyzed (Figure 5C). Interestingly, and contrary to what has been shown by others, we did not find increased JAK/STAT5 signaling pathway activation in the two BCR-ABL-positive cases we studied (data not shown).

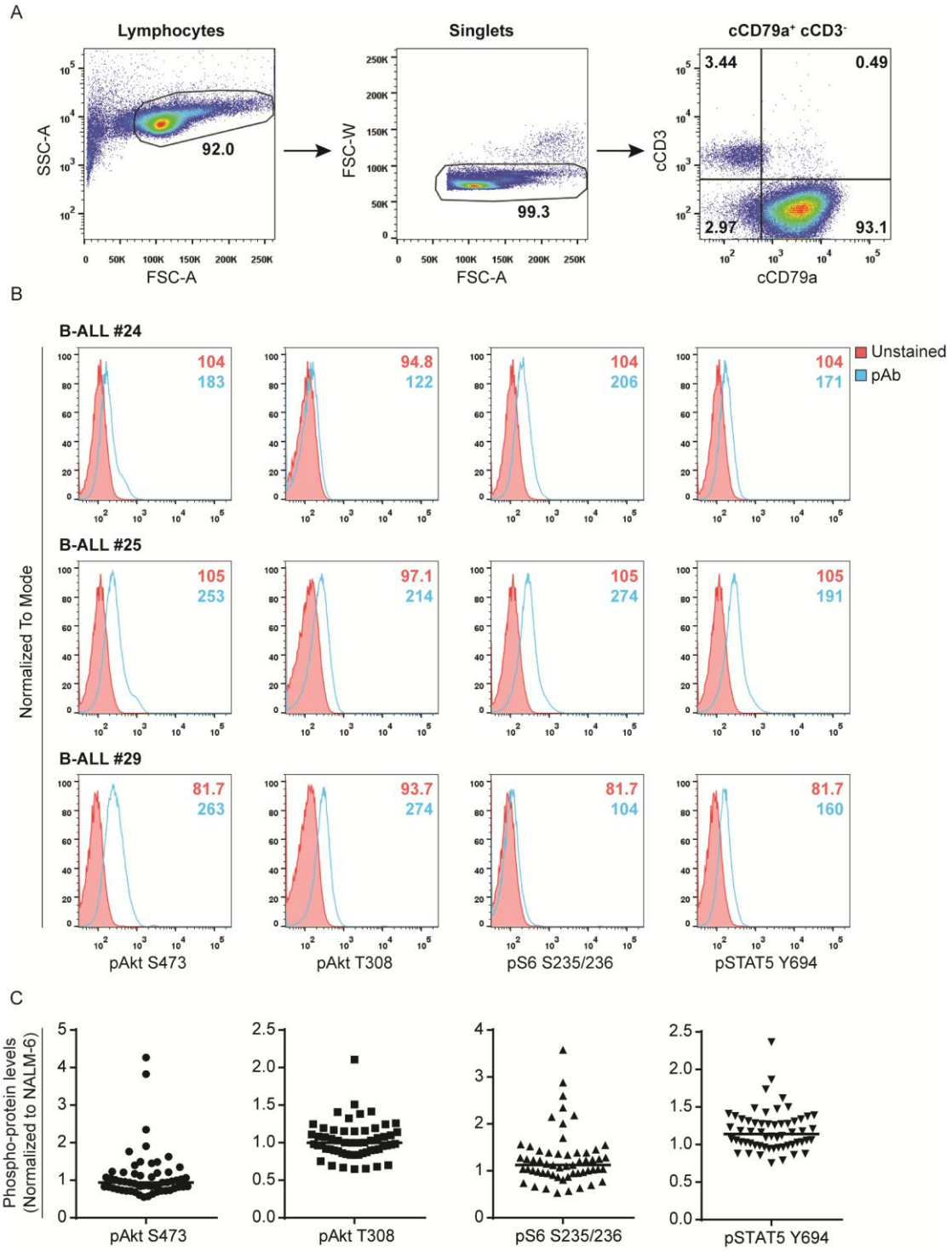


Figure 5. Gating strategy and analysis of signaling pathway activation by phospho-flow cytometry. (A) Lymphocytes were included using a forward scatter area (FSC-A) *vs.* side scatter area (SSC-A) gate. Single cells (Singlets) were then selected on a FSC-A *vs.* forward scatter width (FSC-W) plot to exclude signaling data from doublets. Cytoplasmic CD79a (cCD79a)+ and cytoplasmic CD3 (cCD3)- cells were selected and analysis of individual phospho-proteins at the indicated residues was performed in this cell population. **(B)** Examples of phospho-Akt (S473), phospho-Akt (T308), phospho-S6 (S235/236) and phospho-STAT5 (Y694) histograms for three individual B-ALL patient samples are shown. Background fluorescences from unstained cells were used as negative controls to define positivity in each channel. MFI for each condition (Unstained *vs.* stained for each phospho-antibody) is presented within the histograms. **(C)** Levels of phosphorylated Akt, S6 and STAT5 for all B-ALL samples, after normalization to NALM-6 levels. Points represent individual samples and horizontal bars indicate median.

ALL samples show heterogeneous levels of PI3K/Akt/mTOR and JAK/STAT5 pathway activation in response to IL-7

Previous studies in AML have shown that the response of a leukemic cell to exogenous stimuli may have prognostic value[71]. Moreover, there is evidence indicating that IL-7, which is present in the leukemia microenvironment, can contribute to leukemia progression and drug resistance *in vitro* and *in vivo*[46, 68]. Therefore, we sought to understand whether the response of B-ALL cells to a pro-survival stimulus, such as the one induced by IL-7, may have prognostic value. To this end, we started by analyzing the response of B-ALL cells to IL-7, by examining its effects on specific signaling pathways, namely PI3K/Akt/mTOR and JAK/STAT5.

We cultured primary B-ALL samples for 30 minutes in the presence or absence of IL-7, and, afterwards, evaluated the levels of phosphorylation of Akt, S6 and STAT5, to assess the effects of IL-7 on PI3K/Akt/mTOR and JAK/STAT5 pathways, respectively (Figure 6A). We used the same methodology and gating strategy as described above (Figure 5A). As shown in Figure 6B, the majority of B-ALL samples responded to IL-7 with activation of PI3K/Akt/mTOR, JAK/STAT5 or both pathways. Of note, the level of response varied considerably between patients (Figure 6A and 6B).

Importantly, to validate our phospho-flow results, we have further measured the expression and phosphorylation of Akt, S6 and STAT5 proteins by western blot. This was performed for eight patients, for which we had sufficient biological material. In agreement with what we observed by flow cytometry, similar qualitative results were obtained by western blot. IL-7 stimulation led to the upregulation of STAT5 phosphorylation in all patient samples and of Akt phosphorylation in seven out of eight samples, without affecting total protein levels (Figure 6C). Regarding the phosphorylation levels of S6, IL-7 mildly increased those levels in four out of eight primary samples (Figure 6C). However, in contrast with our phospho-flow analysis, phospho-STAT5 in medium condition was barely detected by western blot (Figure 6C). It is important to refer that although the qualitative tendency was the same using both techniques, the absolute fold changes calculated by each one differed considerably (data not shown).

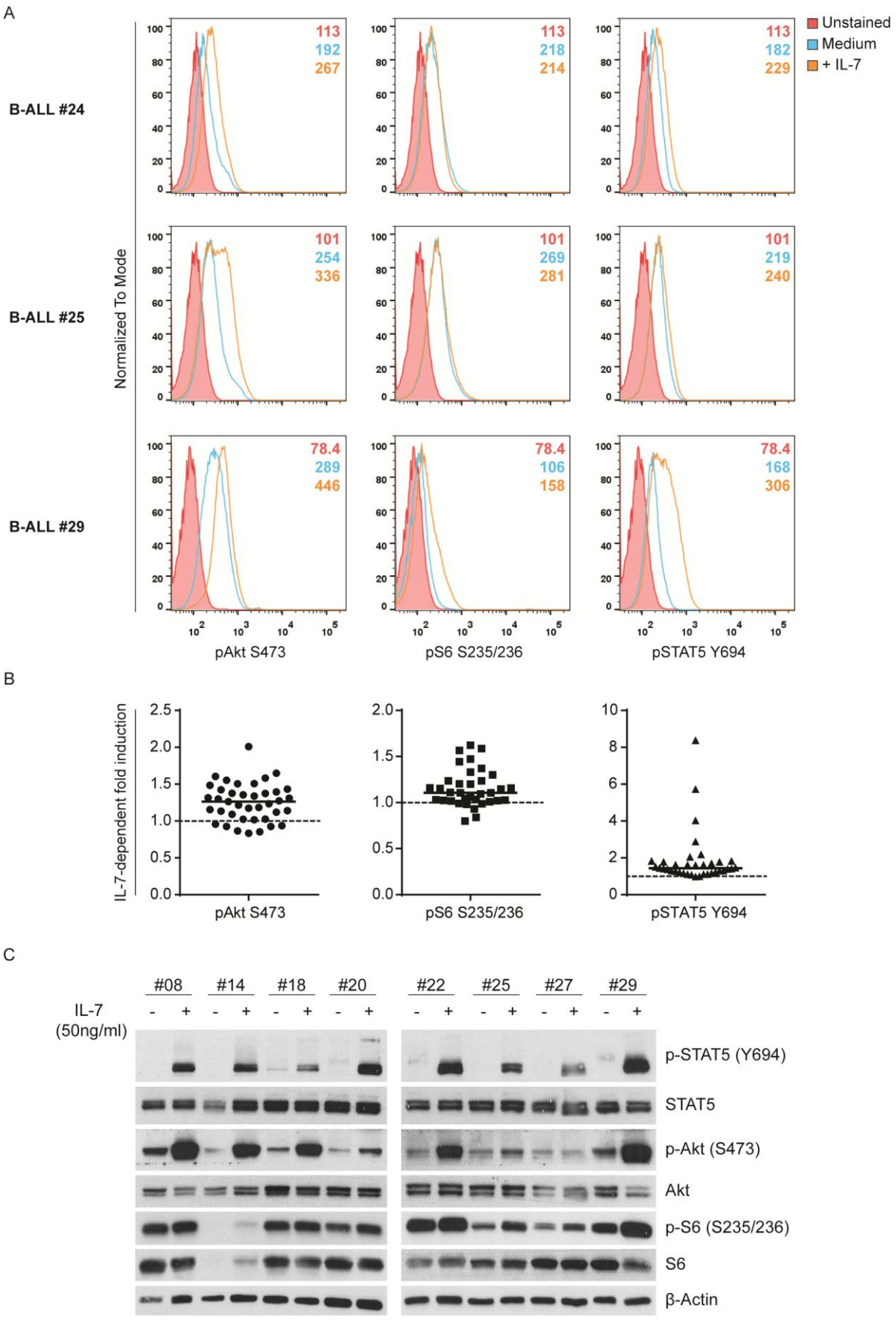

A
B-ALL #24
B-ALL #25
B-ALL #29
Normalized To Mode
Unstained
Medium
+ IL-7
113
192
267
113
218
214
113
182
229
101
254
336
101
269
281
101
219
240
78.4
289
446
78.4
106
158
78.4
168
306
pAkt S473
pS6 S235/236
pSTAT5 Y694
B
IL-7-dependent fold induction
pAkt S473
pS6 S235/236
pSTAT5 Y694
C
#08
#14
#18
#20
#22
#25
#27
#29
IL-7
(50ng/ml)
p-STAT5 (Y694)
STAT5
p-Akt (S473)
Akt
p-S6 (S235/236)
S6
β-Actin

Figure 6. Heterogeneity of IL-7-mediated signaling responses in B-ALL patient samples. (A) The levels of Akt (S473), S6 (S235/235), and STAT5 (Y694) phosphorylation in response to IL-7 stimulation (50ng/ml; 30 minutes) were quantified by flow cytometry analysis using phospho-specific antibodies. Examples of three individual B-ALL patients are presented. Background fluorescences from unstained cells were used as negative controls to define positivity in each channel. MFI for each condition (Unstained in medium condition *vs.* stained for each phospho-antibody in medium or IL-7 conditions) is shown within the histograms. **(B)** Levels of phosphorylated Akt, S6 and STAT5 normalized to medium condition for all B-ALL samples are shown. Points represent individual samples and horizontal bars indicate median. **(C)** Primary B-ALL cells (n=8) were stimulated for 30 minutes with control solution (non-stimulated) or 50ng/ml of IL-7, and levels of expression and phosphorylation of Akt, S6 and STAT5 were analyzed by immunoblotting. β-actin was used as loading control.

High degree of heterogeneity of functional responses to IL-7 and IL-7Rα expression in primary ALL samples

In order to correlate the levels of signaling activation in response to IL-7 with the functional output, we carried out functional analyses in response to IL-7. It is important to have in mind that IL-7 has been implicated in promoting both viability and cell cycle progression in leukemic cells. We started by analyzing the effects of IL-7 on cell viability. Thus, we cultured primary ALL cells in the presence or not of IL-7 and, at pre-defined time points, we performed flow cytometry analysis of Annexin V/7AAD staining and FSCxSSC discrimination to evaluate the impact of IL-7 on viability and cell size (an indirect measure of proliferation), respectively (Figure 7A and 7B). The majority of the patient samples were sensitive to IL-7 addition, although to different extents. Overall, IL-7 incubation led to a significant increase in the frequency of live cells (Annexin V and 7AAD double-negative) and a decrease in the frequency of early apoptotic cells (Annexin V positive and 7AAD negative) and late apoptotic or necrotic cells (Annexin V and 7AAD double-positive) (Figure 7B). Also, it was observed a significant increase in cell size of primary ALL cells after incubation with IL-7, indirectly hinting on an increase in cell proliferation (Figure 7B). Whenever possible (n=11), we also directly determined the effects of IL-7 stimulation on cell proliferation by thymidine incorporation assays. In general, leukemic cells responded to IL-7 by increasing their proliferation, although to different levels (Figure 7B). These findings support the notion that IL-7 stimulation promotes both cell viability and cell proliferation of B-ALL samples, albeit in a heterogeneous way. When we compared the molecular and functional analysis, we did not find any correlation between the levels of PI3K/Akt/mTOR or JAK/STAT5 pathway activation by phospho-flow and the functional outcome on proliferation and viability (data not shown).

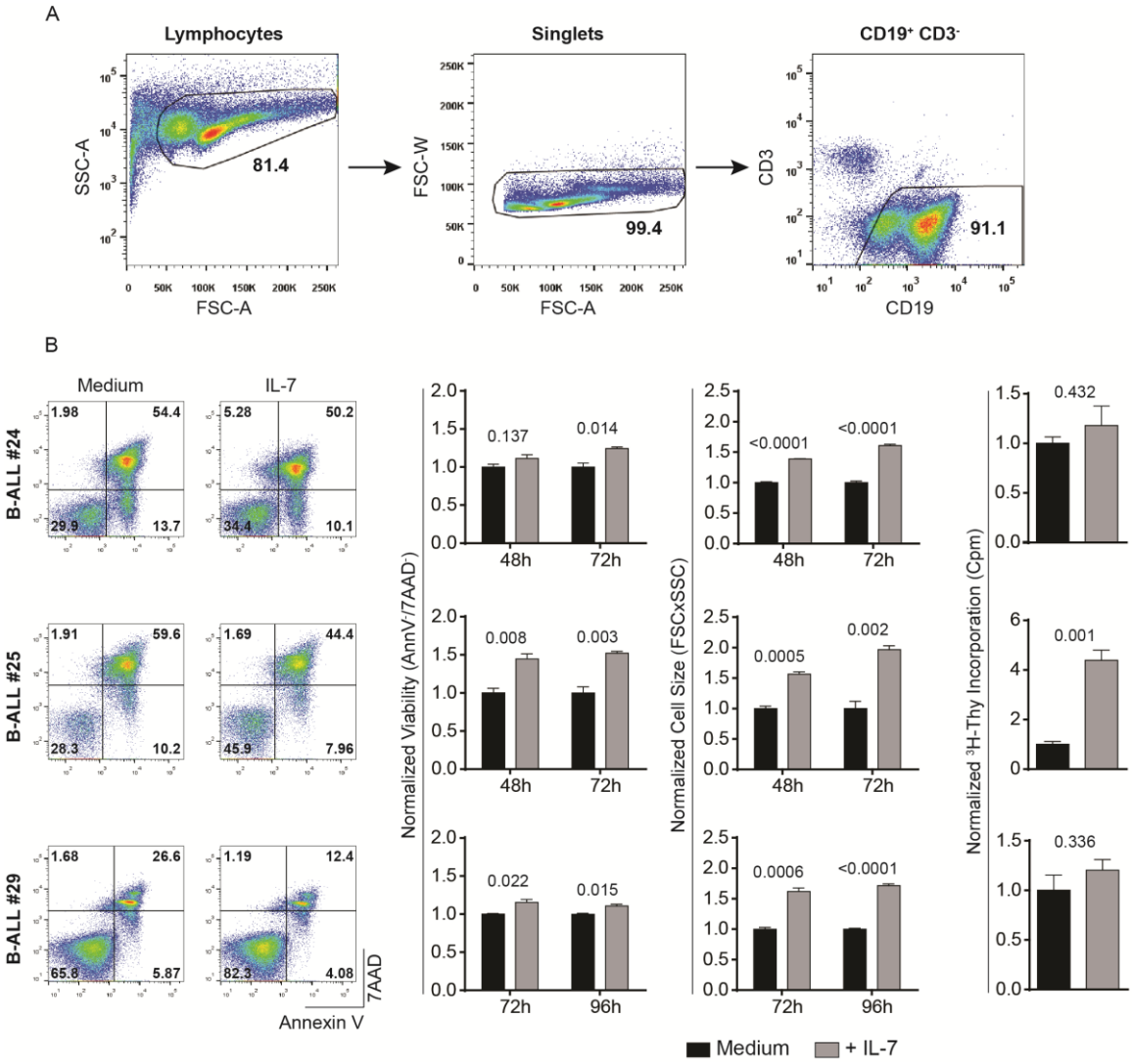


Figure 7. Heterogeneity of functional responses to IL-7 in B-ALL patient samples. (A) Lymphocytes were included using a FSC *vs.* SSC gate and single cells (Singlets) were then selected on a FSC-A *vs.* FSC-W plot to exclude signaling data from doublets. Surface CD19+ and surface CD3- cells were selected and analysis of viability and cell size was performed in this cell population. **(B)** Primary leukemic cells collected at diagnosis were cultured with medium alone or 10ng/ml of IL-7 and analyzed for cell viability and cell size increase at two different time points, according to each patient. Viability and cell size were evaluated by flow cytometry analysis of Annexin V/7AAD staining and FSCxSSC discrimination, respectively. The percentage of live cells (bottom left), early apoptotic cells (bottom right), and late apoptotic or necrotic cells (top right) is indicated in the respective quadrants. Thymidine incorporation assays were also performed to directly determine the impact of IL-7 on cell proliferation. Results are presented as normalized mean±SEM to medium conditions. Statistical analysis from triplicates was performed using Student's t test and *p* values are shown in the graphics. Examples of three representative ALL patients are presented.

Next, we assessed the expression of the IL-7Rα (CD127) in B-ALL blast cells *ex vivo* (0h) (Figure 8A and 8B). This expression was evaluated in order to correlate it with the molecular (signaling pathway activation) and functional (viability, cell growth) outcomes after IL-7 *in vitro* stimulation. We also compared the IL-7Rα expression levels with clinical parameters, such as age, WBC counts, maturation stage and MRD status. As

observed for the levels of activation, IL-7Rα expression is highly heterogeneous among the diagnostic ALL samples (Figure 8C).

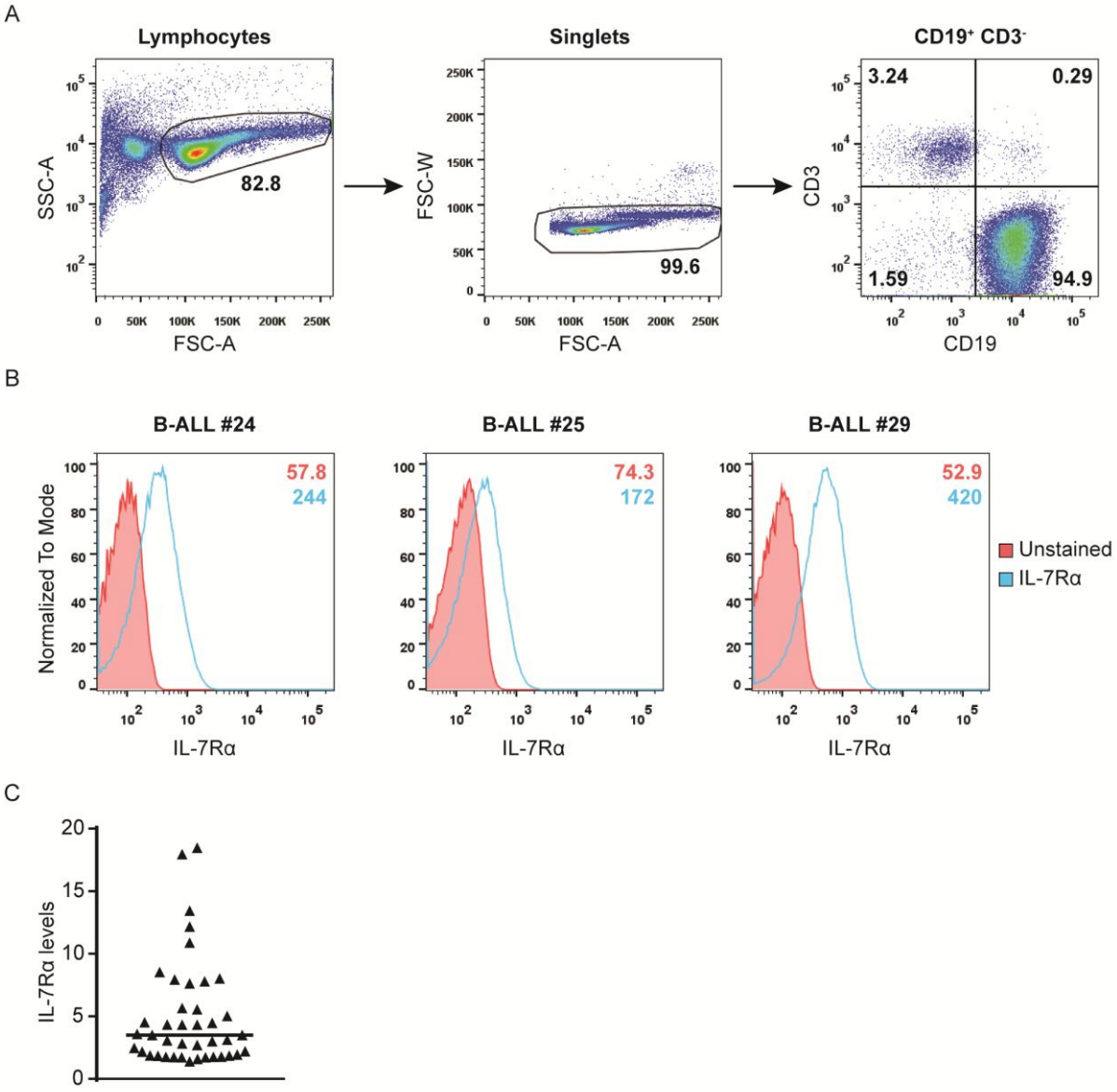


Figure 8. IL-7Rα expression in primary B-ALL cells. (A) Lymphocytes were included using a FSC *vs.* SSC gate and single cells (Singlets) were then selected on a FSC-A *vs.* FSC-W plot to exclude signaling data from doublets. Surface CD19+ and surface CD3- cells were selected and analysis of IL-7Rα protein levels was performed in this cell population. **(B)** Levels of IL-7Rα expression were quantified by flow cytometry using a specific antibody. Examples of histograms for three individual B-ALL patient samples are shown. Background fluorescences from unstained cells were used as negative controls to define positivity in each channel. MFI for each condition (Unstained *vs.* stained for IL-7Rα) is presented within the histograms. **(C)** Levels of IL-7Rα expression for all B-ALL samples. Points represent individual samples and horizontal bar indicates median.

By correlating the IL-7Rα expression levels with the phosphorylation levels of Akt, S6 and STAT5 in response to IL-7 stimulation, we found that higher expression of the receptor does not translate into increased responses (Figure 9A). In fact, surprisingly, when looking at the levels of phosphorylation of STAT5, we observed that some patient samples with lower IL-7Rα expression responded to IL-7 with higher upregulation of STAT5 phosphorylation than the others (Figure 9A). In terms of functional outcome, our results demonstrated that IL-7-mediated cell viability, as well as cell proliferation, do not significantly associate with IL-7Rα expression levels (Figure 9B). The comparison with clinical parameters gave us a trend suggesting that children older than 10 years of age have higher expression levels of the receptor (Figure S1).

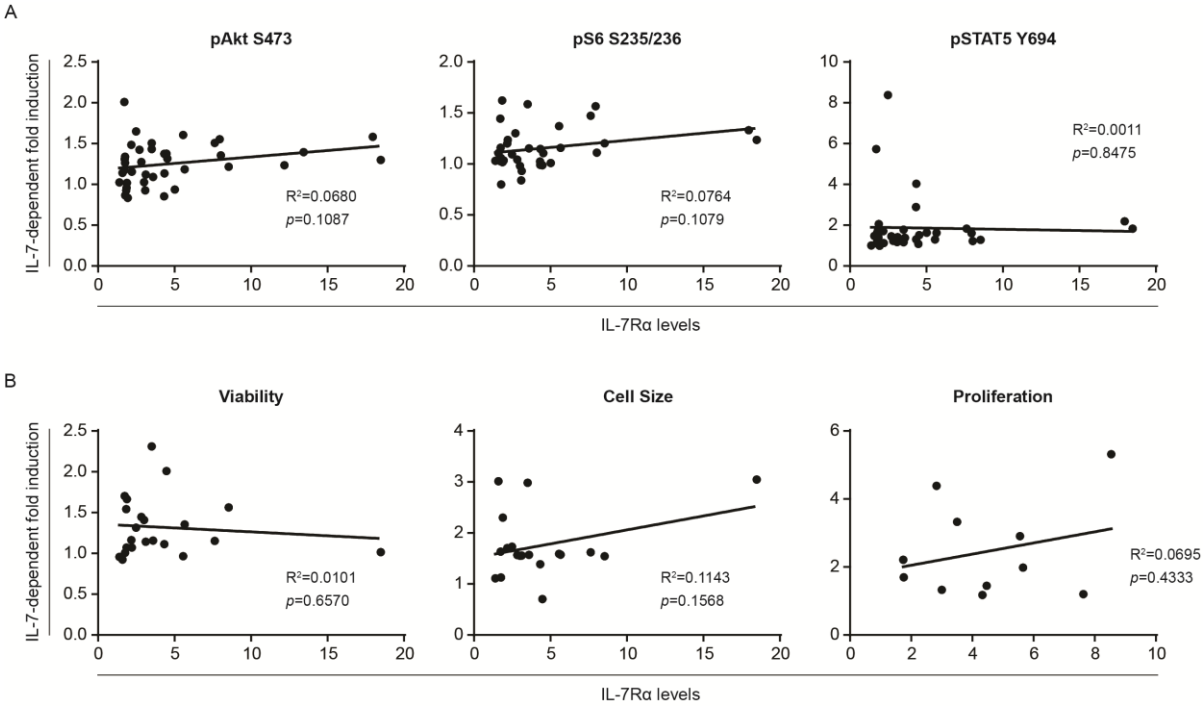


Figure 9. IL-7Rα expression levels do not correlate with signaling pathway activation or functional outcomes in pediatric B-ALL samples. (A) Correlation between IL-7Rα expression levels and IL-7-stimulated levels of Akt (S473), S6 (S235/236) and STAT5 (Y694) phosphorylation. **(B)** Correlation between IL-7Rα expression levels and IL-7-induced viability, cell size and proliferation. Statistical analysis was performed by linear regression. R square and *p* values are shown in the graphics. Points represent individual samples.

Levels of Akt and S6 phosphorylation associate with WBC counts

There is a clear demand to establish new parameters capable of sub-classifying subtypes of B-ALL and predicting response to treatment. Indeed, they may have a significant impact in therapeutic decisions and open new routes for molecular investigation and targeted treatment, which are both important to reduce therapy resistance and disease relapse.

To determine whether basal (Figure 5) and/or IL-7-induced (Figure 6) levels of activation of PI3K/Akt/mTOR and JAK/STAT5 pathways may have prognostic value, we correlated the activation levels of each pathway with well establish clinical features with known prognostic value, such as: a) age and WBC counts at diagnosis; and b) MRD at the end of induction therapy. As pointed out earlier, this is one of the most robust independent prognostic markers in ALL. We also associated the levels of activation with the B-ALL maturation stage (EGIL classification). We did not find evidence for significant associations between basal or IL-7-stimulated activation levels of both signaling pathways with age or maturation stage (Figure 10). Remarkably, higher basal levels of phosphorylation of S6 on S235/236 and Akt on S473, but not Akt on T308 and STAT5 on Y694, significantly correlated with higher WBC counts (Figure 11A). Regarding IL-7-stimulated levels, although non-significant, we observed a trend suggestive of a correlation between higher responses to IL-7 at the signaling level with lower WBC counts (Figure 11B). None of the basal or IL-7-induced phospho-protein levels analyzed were significantly associated with MRD status (Figure 12).

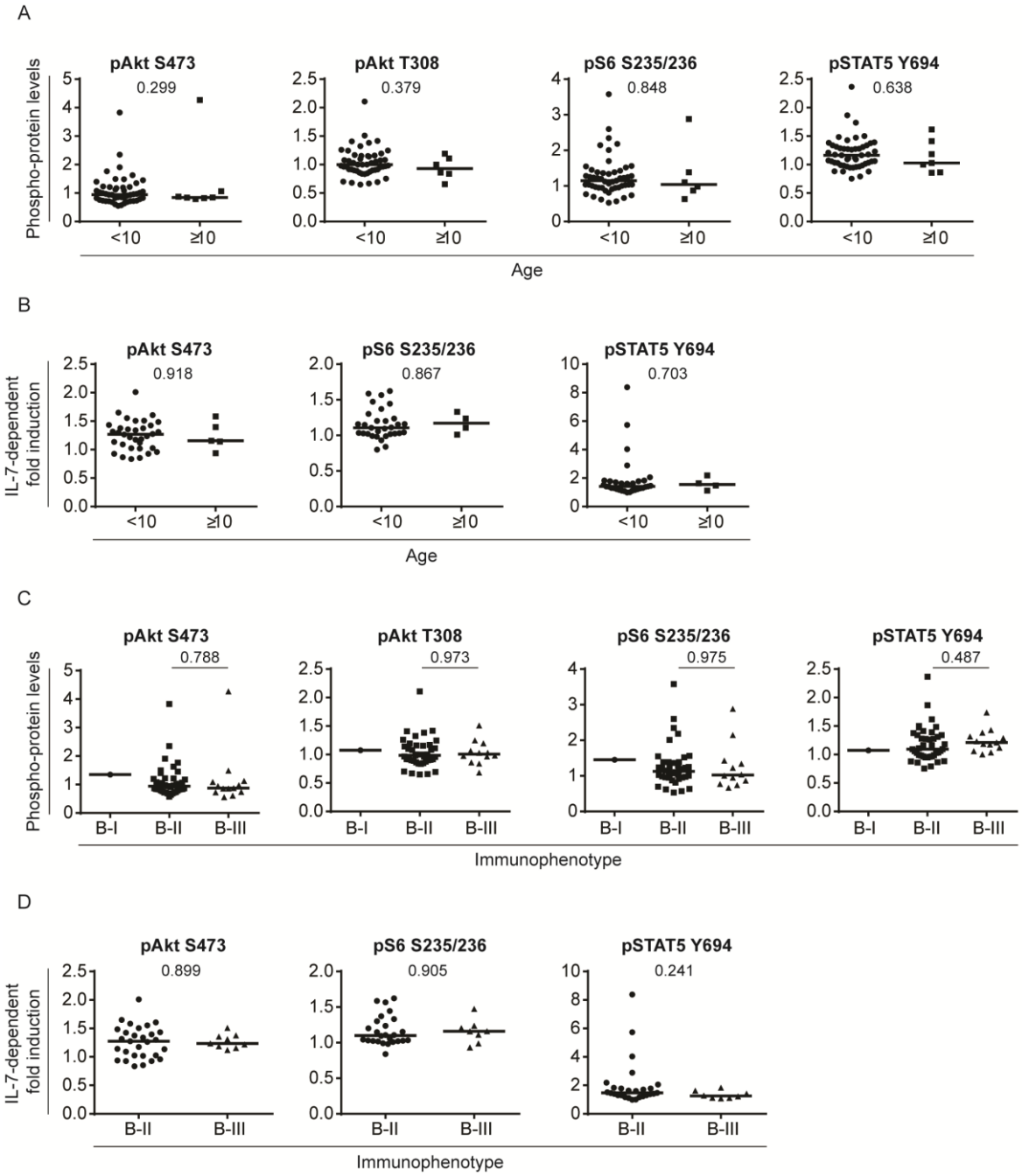


Figure 10. Basal or IL-7-stimulated levels of activation of PI3K/Akt/mTOR and JAK/STAT5 pathways do not appear to correlate with age or maturation stage in pediatric B-ALL patients. Association between basal levels of Akt (S473 and T308), S6 (S235/236) and STAT5 (Y694) phosphorylation and age **(A)** or B-ALL maturation stage, as defined by EGIL classification (B-I, B-II and B-III) **(C)**. Basal levels were normalized to those of the reference cell line, NALM-6. Association between IL-7-induced levels of Akt (S473), S6 (S235/236) and STAT5 (Y694) phosphorylation and age **(B)** or B-ALL maturation stage **(D)**. Statistical analysis was performed by Student's t test and *p* values are shown in the graphics. Points represent individual samples and horizontal bars indicate median.

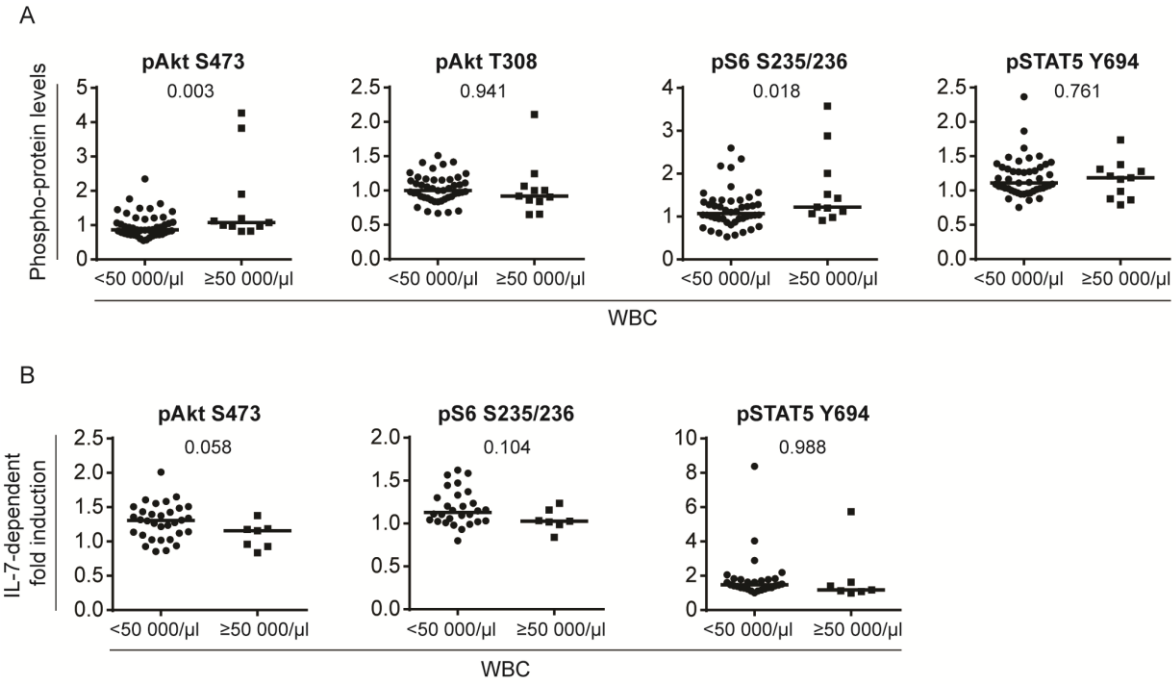


Figure 11. Basal phosphorylation levels of S6 on S235/236 and Akt on S473 associate with WBC counts in B-ALL patient samples. (A) Association between basal levels of phosphorylation of Akt (S473 and T308), S6 (S235/236) and STAT5 (Y694) and WBC counts. Levels were normalized to those of NALM-6, the reference cell line. **(B)** Association between IL-7-stimulated levels of phosphorylation of Akt (S473), S6 (S235/236) and STAT5 (Y694) and WBC counts. Statistical analysis was performed using Student's t test and *p* values are shown in the graphics. Points represent individual samples and horizontal bars indicate median.

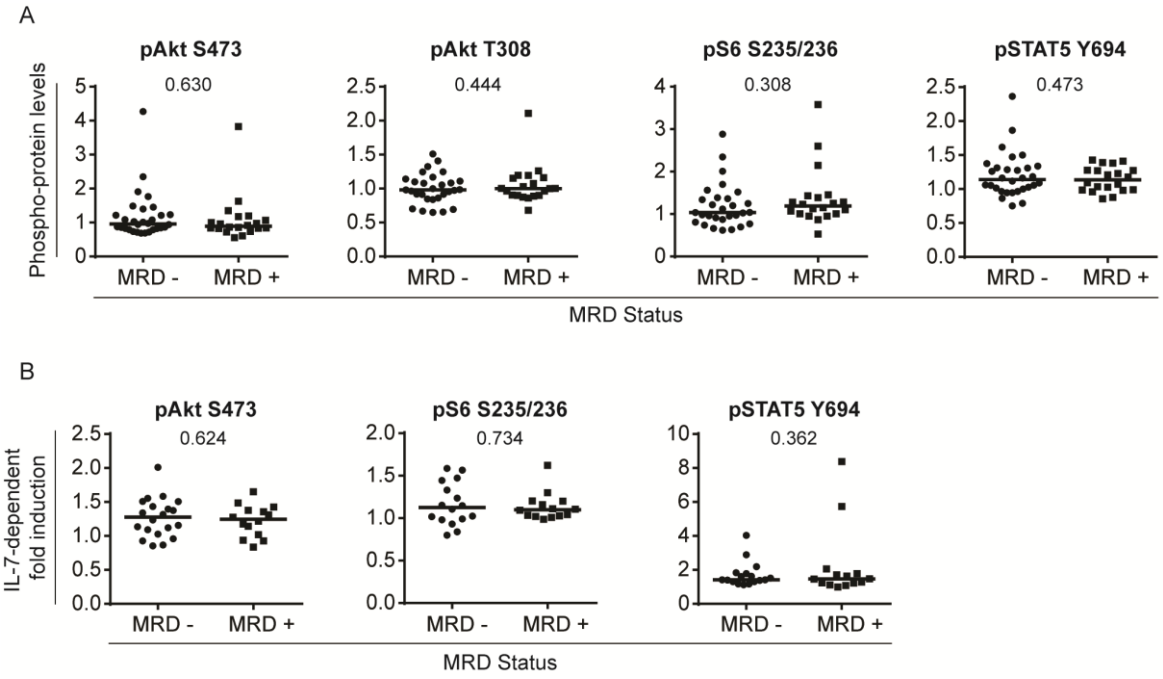


Figure 12. Basal or IL-7-stimulated levels of activation of PI3K/Akt/mTOR and JAK/STAT5 pathways do not associate with MRD status in pediatric B-ALL patients. (A) Association between basal levels of Akt (S473 and T308), S6 (S235/236) and STAT5 (Y694) phosphorylation and MRD status after induction therapy. Levels were normalized to those of NALM-6, the reference cell line. **(B)** Association between IL-7-induced levels of Akt (S473), S6 (S235/236) and STAT5 (Y694) phosphorylation and MRD status. Statistical analysis was performed using Student's t test and *p* values are shown in the graphics. Points represent individual samples and horizontal bars indicate median.

Discussion

In the current thesis, we sought to study the prognostic value of the activation levels of JAK/STAT5 and PI3K/Akt/mTOR pathways, basally and upon IL-7 stimulation, in pediatric B-ALL samples using phospho-flow cytometry. The value of this methodology as a tool to predict patient responses to therapy was demonstrated by Nolan and colleagues[71]. These authors have evaluated the activation status of oncogenic signaling pathways at the single cell level using this approach in primary AML patient samples, and they observed a correlation between particular signal transduction profiles and resistance to chemotherapy. The other reason determinant when choosing phospho-flow cytometry for our studies was the feasibility of applying this technique to actual clinical diagnostics, since flow cytometry is already used in the clinic to subclassify leukemia patients based on their immunophenotype and for MRD detection. Of note, another advantage of this method is that it can be applied even when restricted cell numbers are available.

Here, we show that the basal levels of JAK/STAT5 and PI3K/Akt/mTOR pathway activation vary considerably among pediatric B-ALL patients. Similar results have been previously reported, both in AML and adult B-ALL patients, demonstrating the significant heterogeneity at the signaling level of patient samples[70, 71].

Given that it is very difficult to obtain BM biopsies from healthy donors, we were not able to compare the B-ALL blast cells from leukemia patients with normal primary BM cells – and this was not, in fact, required for our goals. Nonetheless, we were able to compare the levels of pediatric ALL patients with the levels of adult patients, known to display higher levels of activation of these pathways in comparison with healthy individuals ([70] and J.T. Barata *et al.* unpublished data). Thus, since the activation levels of pediatric B-ALL cells were higher than the levels of adult B-ALL samples (Figure S2), we speculate that pediatric B-ALL cells likely present higher levels of activation of PI3K/Akt/mTOR pathway than their normal counterparts.

In contrast with previous studies that show that STAT5 is activated downstream of BCR-ABL translocation[59, 61], we observed that the only two Ph-positive ALL cases of our cohort have phosphorylation levels of STAT5 comparable to those of the majority of the patients. We are aware that, given the very small number of patients that we have studied, such finding is not conclusive. Moreover, some of the Ph-negative patients display higher levels of STAT5 phosphorylation, suggesting that these patients could be integrated into the recently created BCR-ABL-negative Ph-like ALL subgroup. Whether high STAT5 phosphorylation may constitute a robust marker of this subgroup, which is associated with poor prognosis[72, 73], remains to be addressed.

Regarding the PI3K/Akt/mTOR pathway, we found that some patients have higher basal levels of Akt and S6 phosphorylation than others. Whether this can be explained by the posttranslational inactivation of the tumor suppressor PTEN, which is a very frequent event in both T-cell[66] and adult B-cell ALL patients[70], needs to be analyzed. Another possibility is that patients with higher levels of basal activation of one or both pathways may have gain-of-function mutations in the IL-7Rα, as demonstrated in T-ALL and B-ALL[50, 56]. Of note, we have not looked at the mutational status of this receptor, since Shochat and his colleagues showed that it is a rare event in childhood B-ALL, occurring in less than 1% of patients without any additional abnormality[50].

Also concerning the basal levels, it would be worthy to measure the plasma IL-7 levels of our cohort of B-ALL patients and correlate them with the basal levels of signaling pathway activation. We can speculate that patients with higher plasma IL-7 levels are the ones with higher basal phosphorylation levels of Akt, S6 and STAT5 *ex vivo*. Furthermore, those plasma levels can also impact, positively or negatively, on the IL-7 responsiveness of B-ALL cells *in vitro*.

We have further verified that IL-7 stimulation of primary cells triggers, as expected, the activation of both signaling pathways in the majority of the patients[31, 48]. Nonetheless, the IL-7-induced activation of Akt, S6 and STAT5 phospho-proteins differed significantly between patient samples. Others have observed this variability in AML samples in response to other cytokines, such as Flt3L, granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-3 and interferon gamma (IFNγ)[71]. Given the focus of our lab on T-ALL research and on normal human T cell development, it would be very interesting to also investigate IL-7-induced signaling in tumor-infiltrating T cells in the context of B-ALL, and compare these results with those of T cells in the periphery (non tumor-associated).

To validate our technique, we also looked at the response to IL-7 by western blot in eight patients, and we tried to establish a correlation between these results with those obtained by intracellular phospho-protein staining for flow cytometry. Immunoblotting and flow cytometry are two distinct methodologies with different sensitivities and intrinsic errors, being the last one the best to analyze several signaling cascades in the same cell in a quick and efficient manner. Nevertheless, it has been previously described that phospho-specific flow cytometry analyses are well correlated with western blotting[74]. Overall, our results using flow cytometry are closely related to those obtained by western blot, in a qualitative way. However, when we compare absolute fold changes between both techniques, they differed considerably. This is well exemplified by the differences

obtained in the levels of STAT5 phosphorylation. As shown in Figure 6, while we observe basal phospho-STAT5 in medium conditions by phospho-flow cytometry, it is barely detected by western blot. Consequently, the IL-7-dependent fold change for this phospho-protein in the western blot is much higher than by flow cytometry.

We have shown that IL-7 increases viability and promotes cell proliferation of primary T-ALL cells, acting mainly through the activation of the PI3K/Akt/mTOR pathway[31, 43-45]. In B-ALL, the proliferative effects of IL-7 stimulation have been also demonstrated by Touw *et al.*[47]. However, this study analyzed a very limited number of patients (n=5) and the response to IL-7 was not consistent in all patients. In agreement, we observed that IL-7 promotes viability and proliferation of primary B-ALL cells, although to different extents. Nonetheless, and contrary to what was observed in T-ALL cells, we did not find a significant association between IL-7-induced higher levels of activation of the PI3K/Akt/mTOR pathway and higher IL-7-mediated viability and proliferation (data not shown). It is important to mention that this correlation was established using a small number of patients (n=22). Thus, further studies with a higher number of primary B-ALL samples are necessary to define the precise role of PI3K/Akt/mTOR pathway in IL-7-induced viability and proliferation of primary B-ALL cells.

We also evaluated the expression levels of IL-7Rα in primary cells, in order to correlate them with both molecular and functional outcomes. We found highly heterogeneous levels between patients. Unfortunately, and as explained above, we were not able to compare the expression levels of the receptor in B-ALL blast cells with those of primary BM cells from healthy individuals. However, there is evidence that IL-7Rα expression is higher in adult pre-B ALL patients than healthy donors, as it was demonstrated by Sasson and others[49]. The authors also analyzed the intracellular expression of the cell-cycle protein Ki-67 and the anti-apoptotic protein Bcl-2 in the pre-B-ALL cells and observed that it was elevated in those cells expressing the IL-7Rα. So, they confirmed a possible association of IL-7Rα expression with a more active proliferative state of leukemic cells. In contrast, our results showed that cell proliferation, as well as cell viability, do not correlate with IL-7Rα expression levels. Similar results were obtained when we compared the same levels with signaling pathway activation upon IL-7 stimulation. Since IL-7Rα expression levels can be easily detected by flow cytometry, and being this technique currently used in clinical diagnostic laboratories, we decided to correlate those levels with clinical parameters with known prognostic value (age, WBC counts, and MRD status) and with maturation stage, in order to address their prognostic significance. Overall, albeit non-significant, we found a trend suggesting that children older than 10 years of age, known to have a poor prognosis, have higher levels of

expression of this receptor (Figure S1). Further studies with a higher number of samples are required to understand whether IL-7Rα may be a possible surrogate marker of B-ALL cases with poor prognosis.

Finally, we correlated the basal and IL-7-induced levels of activation of PI3K/Akt/mTOR and JAK/STAT5 pathways with the clinical features mentioned above. Since all patients analyzed are from a single institution and treated with the same therapeutic protocol, it was possible to avoid biases in the statistical analysis performed. We did not find any significant correlation between basal or IL-7-stimulated activation levels of both pathways in what regards to age, maturation stage or MRD status. Interestingly, higher basal levels of phosphorylation of S6 on S235/236 and Akt on S473, but not on T308, appeared to associate with higher WBC counts, arguing that there may be an association of high phosphorylation levels of these proteins with high risk. This result also suggests that two independent mechanisms (regarding S473 and T308 phosphorylation) lead to Akt activation in B-ALL with different biological outcomes, which can be a relevant question to be evaluated. The fact that we only found significant associations with WBC counts may be because the number of patients that we analyzed is not enough or because this clinical parameter is more associated with high risk rather than poor prognosis, as a consequence of the current therapeutic regimens.

We also would like to correlate the basal and IL-7-induced levels of activation of PI3K/Akt/mTOR and JAK/STAT5 pathways with other clinical parameters, especially those related to survival rates (3-year and 5-year event-free and overall survival), as soon as we have access to those data.

To the best of our knowledge, no studies so far have determined the prognostic value of PI3K/Akt/mTOR and JAK/STAT5 signaling pathway activation in B-ALL using phospho-flow cytometry. There is, however, one recent study where the levels of phosphorylation of Akt on serine 473 were analyzed by western blot in a cohort of 21 pediatric B-ALL patients[75]. After correlation of those levels with prognostic features, such as overall survival and relapse-free survival, the authors observed that Akt phosphorylation is associated with an unfavorable prognosis. This protein is also activated in patients with poor response to induction therapy[75].

Conclusion

Overall, our results suggest a positive association of high constitutive levels of phosphorylation of S6 on S235/236 and Akt on S473 with high risk, which is commonly associated with poor prognosis. Additional studies with a higher number of primary B-ALL samples, which we are currently performing, are required to strength and validate our results and to clarify the real prognostic value of these critical pathways. In the present thesis, we also provided evidence for heterogeneous basal activation of both pathways in primary ALL cells. We also showed that the majority of B-ALL patients tend to respond to IL-7 by up-regulating both signaling pathways, as well as by increasing cell viability and cell proliferation. Nonetheless, it remains to be addressed which of the two pathways, or if both, are involved in the observed functional outcomes.

In conclusion, the completion of the work here described will enable us to determine the prognostic significance of PI3K/Akt/mTOR and JAK/STAT5 signaling pathways in the context of pediatric B-ALL. It can also help validating the value of phospho-flow cytometry in identifying patients with different prognosis, thus speeding up the use of this tool in clinical diagnostics.

References

1. Fuxa, M. & Skok, J.A. Transcriptional regulation in early B cell development. *Curr Opin Immunol* **19**, 129-36 (2007).
2. Bertrand, F.E., 3rd et al. Ig D(H) gene segment transcription and rearrangement before surface expression of the pan-B-cell marker CD19 in normal human bone marrow. *Blood* **90**, 736-44 (1997).
3. Davi, F. et al. Early onset of immunoglobulin heavy chain gene rearrangements in normal human bone marrow CD34+ cells. *Blood* **90**, 4014-21 (1997).
4. Dworzak, M.N., Fritsch, G., Froschl, G., Printz, D. & Gadner, H. Four-color flow cytometric investigation of terminal deoxynucleotidyl transferase-positive lymphoid precursors in pediatric bone marrow: CD79a expression precedes CD19 in early B-cell ontogeny. *Blood* **92**, 3203-9 (1998).
5. Loken, M.R., Shah, V.O., Dattilio, K.L. & Civin, C.I. Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development. *Blood* **70**, 1316-24 (1987).
6. LeBien, T.W. et al. Multiparameter flow cytometric analysis of human fetal bone marrow B cells. *Leukemia* **4**, 354-8 (1990).
7. Ghia, P. et al. Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci. *J Exp Med* **184**, 2217-29 (1996).
8. Scott, E.W., Simon, M.C., Anastasi, J. & Singh, H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265**, 1573-7 (1994).
9. Georgopoulos, K. et al. The Ikaros gene is required for the development of all lymphoid lineages. *Cell* **79**, 143-56 (1994).
10. DeKoter, R.P., Lee, H.J. & Singh, H. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity* **16**, 297-309 (2002).
11. Schebesta, M., Heavey, B. & Busslinger, M. Transcriptional control of B-cell development. *Curr Opin Immunol* **14**, 216-23 (2002).
12. Nagasawa, T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* **6**, 107-16 (2006).
13. Welner, R.S., Pelayo, R. & Kincade, P.W. Evolving views on the genealogy of B cells. *Nat Rev Immunol* **8**, 95-106 (2008).
14. Clark, M.R., Mandal, M., Ochiai, K. & Singh, H. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nat Rev Immunol* **14**, 69-80 (2014).
15. Zhou, Y. et al. Advances in the molecular pathobiology of B-lymphoblastic leukemia. *Hum Pathol* **43**, 1347-62 (2012).
16. Mullighan, C.G. et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* **446**, 758-64 (2007).
17. Purizaca, J., Meza, I. & Pelayo, R. Early lymphoid development and microenvironmental cues in B-cell acute lymphoblastic leukemia. *Arch Med Res* **43**, 89-101 (2012).
18. Heidenreich, O. & Vormoor, J. Malignant stem cells in childhood ALL: the debate continues! *Blood* **113**, 4476-7; author reply 4477 (2009).
19. Cobaleda, C. & Sanchez-Garcia, I. B-cell acute lymphoblastic leukaemia: towards understanding its cellular origin. *Bioessays* **31**, 600-9 (2009).
20. Macmahon, B. & Levy, M.A. Prenatal Origin of Childhood Leukemia. Evidence from Twins. *N Engl J Med* **270**, 1082-5 (1964).
21. Pui, C.H., Robison, L.L. & Look, A.T. Acute lymphoblastic leukaemia. *Lancet* **371**, 1030-43 (2008).
22. Bassan, R., Gatta, G., Tondini, C. & Willemze, R. Adult acute lymphoblastic leukaemia. *Crit Rev Oncol Hematol* **50**, 223-61 (2004).

23. Pui, C.H., Carroll, W.L., Meshinchi, S. & Arceci, R.J. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J Clin Oncol* **29**, 551-65 (2011).
24. Kasner, M.T. Novel targets for treatment of adult acute lymphocytic leukemia. *Curr Hematol Malig Rep* **5**, 207-12 (2010).
25. Onciu, M. Acute lymphoblastic leukemia. *Hematol Oncol Clin North Am* **23**, 655-74 (2009).
26. Bene, M.C. et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* **9**, 1783-6 (1995).
27. DeAngelo, D.J. & Pui, C.-H. 491-507 (American Society of Hematology Self-Assessment Program (ASH-SAP), 2013).
28. Vrooman, L.M. & Silverman, L.B. Childhood acute lymphoblastic leukemia: update on prognostic factors. *Curr Opin Pediatr* **21**, 1-8 (2009).
29. Inaba, H., Greaves, M. & Mullighan, C.G. Acute lymphoblastic leukaemia. *Lancet* **381**, 1943-55 (2013).
30. Namen, A.E. et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* **333**, 571-3 (1988).
31. Barata, J.T., Cardoso, A.A. & Boussiotis, V.A. Interleukin-7 in T-cell acute lymphoblastic leukemia: an extrinsic factor supporting leukemogenesis? *Leuk Lymphoma* **46**, 483-95 (2005).
32. Palmer, M.J. et al. Interleukin-7 receptor signaling network: an integrated systems perspective. *Cell Mol Immunol* **5**, 79-89 (2008).
33. Milne, C.D. & Paige, C.J. IL-7: a key regulator of B lymphopoiesis. *Semin Immunol* **18**, 20-30 (2006).
34. Tal, N., Shochat, C., Geron, I., Bercovich, D. & Izraeli, S. Interleukin 7 and thymic stromal lymphopoietin: from immunity to leukemia. *Cell Mol Life Sci* **71**, 365-78 (2014).
35. Sudo, T. et al. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc Natl Acad Sci U S A* **90**, 9125-9 (1993).
36. Grabstein, K.H. et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J Exp Med* **178**, 257-64 (1993).
37. Peschon, J.J. et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**, 1955-60 (1994).
38. von Freeden-Jeffry, U. et al. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-26 (1995).
39. DiSanto, J.P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* **92**, 377-81 (1995).
40. Prieyl, J.A. & LeBien, T.W. Interleukin 7 independent development of human B cells. *Proc Natl Acad Sci U S A* **93**, 10348-53 (1996).
41. Puel, A., Ziegler, S.F., Buckley, R.H. & Leonard, W.J. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genet* **20**, 394-7 (1998).
42. Corfe, S.A. & Paige, C.J. The many roles of IL-7 in B cell development; mediator of survival, proliferation and differentiation. *Semin Immunol* **24**, 198-208 (2012).
43. Barata, J.T. et al. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J Exp Med* **200**, 659-69 (2004).
44. Barata, J.T. et al. IL-7-dependent human leukemia T-cell line as a valuable tool for drug discovery in T-ALL. *Blood* **103**, 1891-900 (2004).
45. Barata, J.T., Cardoso, A.A., Nadler, L.M. & Boussiotis, V.A. Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). *Blood* **98**, 1524-31 (2001).
46. Silva, A. et al. IL-7 contributes to the progression of human T-cell acute lymphoblastic leukemias. *Cancer Res* **71**, 4780-9 (2011).

47. Touw, I. et al. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. *Blood* **75**, 2097-101 (1990).
48. van der Plas, D.C. et al. Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor alpha chain. *Leukemia* **10**, 1317-25 (1996).
49. Sasson, S.C. et al. IL-7 receptor is expressed on adult pre-B-cell acute lymphoblastic leukemia and other B-cell derived neoplasms and correlates with expression of proliferation and survival markers. *Cytokine* **50**, 58-68 (2010).
50. Shochat, C. et al. Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. *J Exp Med* **208**, 901-8 (2011).
51. Jotta, P.Y. et al. Negative prognostic impact of PTEN mutation in pediatric T-cell acute lymphoblastic leukemia. *Leukemia* **24**, 239-42 (2010).
52. Gutierrez, A. et al. High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood* **114**, 647-50 (2009).
53. Bandapalli, O.R. et al. NOTCH1 activation clinically antagonizes the unfavorable effect of PTEN inactivation in BFM-treated children with precursor T-cell acute lymphoblastic leukemia. *Haematologica* **98**, 928-36 (2013).
54. Rane, S.G. & Reddy, E.P. Janus kinases: components of multiple signaling pathways. *Oncogene* **19**, 5662-79 (2000).
55. Jiang, Q. et al. Cell biology of IL-7, a key lymphotrophin. *Cytokine Growth Factor Rev* **16**, 513-33 (2005).
56. Zenatti, P.P. et al. Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. *Nat Genet* **43**, 932-9 (2011).
57. Goetz, C.A., Harmon, I.R., O'Neil, J.J., Burchill, M.A. & Farrar, M.A. STAT5 activation underlies IL7 receptor-dependent B cell development. *J Immunol* **172**, 4770-8 (2004).
58. Malin, S. et al. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. *Nat Immunol* **11**, 171-9 (2010).
59. Malin, S., McManus, S. & Busslinger, M. STAT5 in B cell development and leukemia. *Curr Opin Immunol* **22**, 168-76 (2010).
60. Tasian, S.K. et al. Aberrant STAT5 and PI3K/mTOR pathway signaling occurs in human CRLF2-rearranged B-precursor acute lymphoblastic leukemia. *Blood* **120**, 833-42 (2012).
61. Carlesso, N., Frank, D.A. & Griffin, J.D. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J Exp Med* **183**, 811-20 (1996).
62. Hoelbl, A. et al. Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO Mol Med* **2**, 98-110 (2010).
63. Song, G., Ouyang, G. & Bao, S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* **9**, 59-71 (2005).
64. Clayton, E. et al. A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation. *J Exp Med* **196**, 753-63 (2002).
65. Fruman, D.A. et al. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science* **283**, 393-7 (1999).
66. Silva, A. et al. PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* **118**, 3762-74 (2008).
67. Tesfai, Y. et al. Interactions between acute lymphoblastic leukemia and bone marrow stromal cells influence response to therapy. *Leuk Res* **36**, 299-306 (2012).
68. Brown, V.I. et al. Rapamycin is active against B-precursor leukemia in vitro and in vivo, an effect that is modulated by IL-7-mediated signaling. *Proc Natl Acad Sci U S A* **100**, 15113-8 (2003).
69. Mullighan, C.G. The molecular genetic makeup of acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program* **2012**, 389-96 (2012).

70. Gomes, A.M. et al. Adult B-cell acute lymphoblastic leukemia cells display decreased PTEN activity and constitutive hyperactivation of PI3K/Akt pathway despite high PTEN protein levels. *Haematologica* **99**, 1062-8 (2014).
71. Irish, J.M. et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell* **118**, 217-28 (2004).
72. Roberts, K.G. et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. *Cancer Cell* **22**, 153-66 (2012).
73. Roberts, K.G. et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med* **371**, 1005-15 (2014).
74. Krutzik, P.O. & Nolan, G.P. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A* **55**, 61-70 (2003).
75. Morishita, N. et al. Activation of Akt is associated with poor prognosis and chemotherapeutic resistance in pediatric B-precursor acute lymphoblastic leukemia. *Pediatr Blood Cancer* **59**, 83-9 (2012).

Supplementary Information

Table S1. Immunophenotype and cytogenetics features of B-ALL patient samples.

Patient nº.	Gender	Age (years)	% Blasts	Cytogenetics	Immunophenotype (EGIL Classification)
1	Male	14	90	n.d.	B-II (Common B)
2	Male	3	93	n.d.	B-III (Pre-B)
3	Female	7	95	n.d.	B-III (Pre-B)
4	Male	8	60	n.d.	B-II (Common B)
5	Female	5	92	n.d.	B-II (Common B)
6	Male	7	94	n.d.	B-II (Common B)
7	Female	3	92	n.d.	B-III (Pre-B)
8	Male	5	95	n.d.	B-II (Common B)
9	Female	8	82	n.d.	B-III (Pre-B)
10	Male	2	90	ETV6-RUNX1	B-III (Pre-B)
11	Female	2	85	ETV6-RUNX1	B-III (Pre-B)
12	Male	11	85	n.d.	B-III (Pre-B)
13	Male	7	90	n.d.	B-III (Pre-B)
14	Male	8	81.3	ETV6-RUNX1	B-III (Pre-B)
15	Female	9	95	n.d.	B-III (Pre-B)
16	Male	5	90	ETV6-RUNX1	B-III (Pre-B)
17	Male	2	69	n.d.	B-II (Common B)
18	Male	3	76	-	B-II (Common B)
19	Male	2	96	Hyperdiploidy	B-II (Common B)
20	Female	2	90	ETV6-RUNX1	B-II (Common B)
21	Male	1	70	n.d.	B-II (Common B)
22	Female	3	87	Hyperdiploidy	B-II (Common B)
23	Female	2	92	ETV6-RUNX1; Trisomy 21	B-II (Common B)
24	Male	3	90	Hyperdiploidy	B-II (Common B)
25	Male	2	88	MLL-ENL	B-II (Common B)
26	Male	14	75	n.d.	B-II (Common B)
27	Female	5	83	Hyperdiploidy	B-II (Common B)
28	Male	9	94	n.d.	B-II (Common B)
29	Female	5	95.4	ETV6-RUNX1	B-III (Pre-B)
30	Female	10	90	Hyperdiploidy	B-II (Common B)
31	Male	3	95	ETV6-RUNX1; Trisomy 21	B-II (Common B)
32	Female	2	n.d.	-	n.d.
33	Female	3	84	ETV6-RUNX1	B-II (Common B)
34	Female	11	95	n.d.	B-II (Common B)
35	Male	3	95	n.d.	B-I (Pro-B)
36	Male	6	95	ETV6-RUNX1	B-II (Common B)

Patient nº.	Gender	Age (years)	% Blasts	Cytogenetics	Immunophenotype (EGIL Classification)
37	Female	4	88	ETV6-RUNX1	n.d.
38	Female	4	85	ETV6-RUNX1	B-II (Common B)
39	Male	3	92	n.d.	B-II (Common B)
40	Female	3	80	ETV6-RUNX1	B-II (Common B)
41	Female	13	91	E2A-PBX1	B-III (Pre-B)
42	Female	3	94	ETV6-RUNX1	B-II (Common B)
43	Male	3	95	n.d.	B-II (Common B)
44	Female	2	92	Hyperdiploidy	B-II (Common B)
45	Male	3	94	n.d.	B-II (Common B)
46	Male	3	86	n.d.	B-II (Common B)
47	Female	4	86	ETV6-RUNX1	B-II (Common B)
48	Male	2	81	Hyperdiploidy	B-II (Common B)
49	Male	3	88	Hyperdiploidy	B-II (Common B)
50	Female	2	90	BCR-ABL	B-II (Common B)
51	Female	2	96.1	ETV6-RUNX1	B-II (Common B)
52	Male	6	95	ETV6-RUNX1	B-II (Common B)
53	Male	13	54	E2A-PBX1	B-II (Common B)
54	Male	4	n.d.	ETV6-RUNX1	B-II (Common B)
55	Female	7	80	BCR-ABL	B-II (Common B)
56	Male	3	n.d.	Hyperdiploidy	n.d.
57	Male	1	90	Hyperdiploidy	B-II (Common B)
58	Male	2	63	-	B-II (Common B)

-: not detected; n.d.: not determined

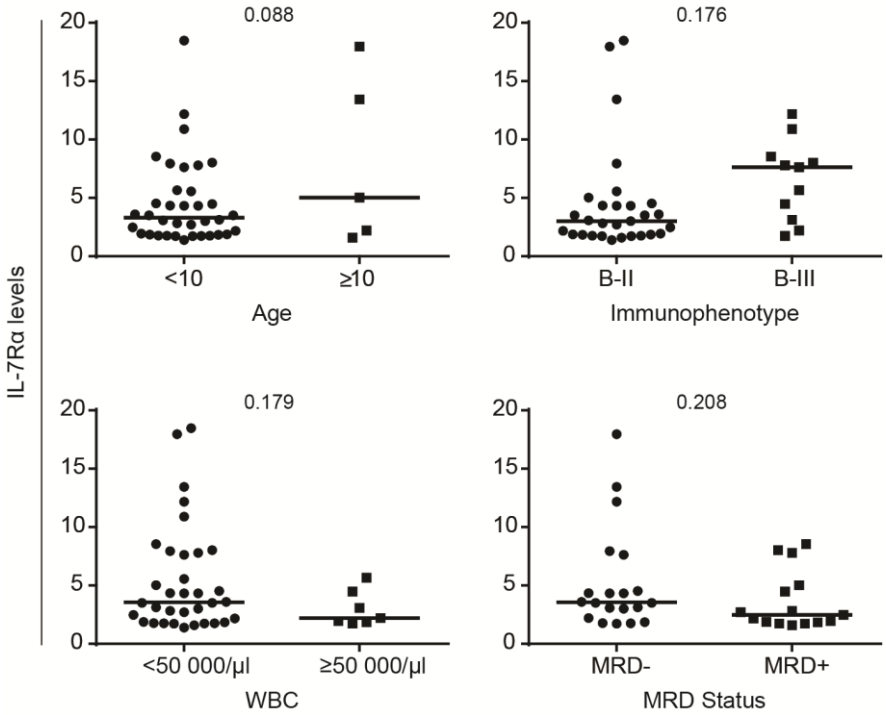


Figure S1. IL-7Rα expression levels do not associate with age, maturation stage, WBC counts or MRD status in pediatric B-ALL samples. Association between IL-7Rα expression levels and clinical parameters: age, maturation stage, WBC counts and MRD status. Statistical analysis was performed by Student's t test and *p* values are shown in the graphics. Points represent individual samples and horizontal bars indicate median.

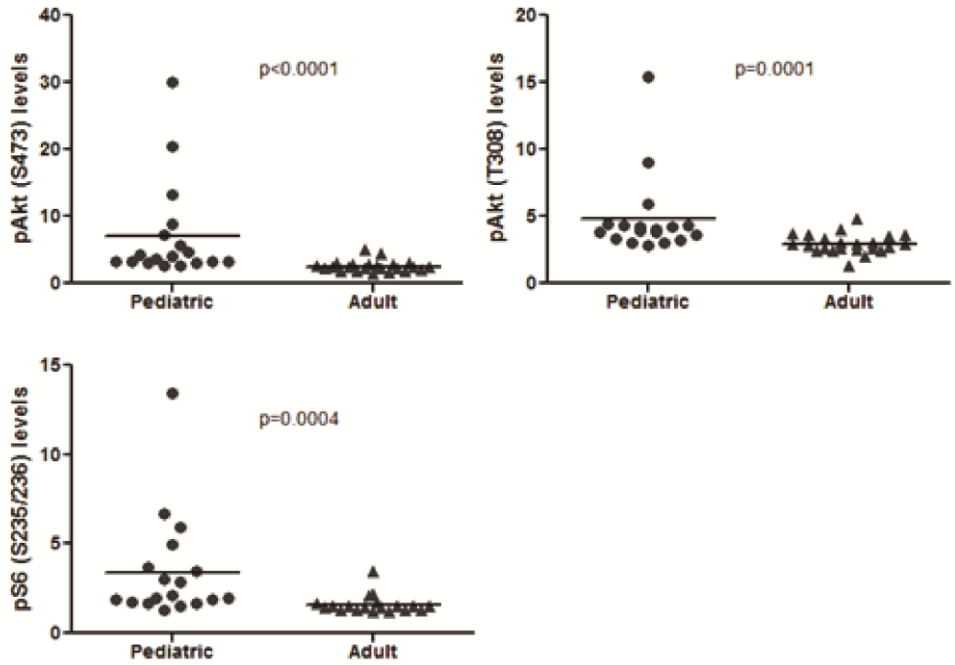


Figure S2. Pediatric B-ALL cases display significantly higher levels of PI3K/Akt/mTOR pathway activation than adults. Levels of phosphorylated Akt (S473), Akt (T308) and S6 (S235/236) in bone marrow cells from pediatric and adult B-ALL samples were quantified by flow cytometry analysis using phospho-specific antibodies. Points represent individual samples and horizontal bars denote median. Statistical analysis was performed by two-tailed Mann Whitney test and *p* values are shown in the graphics. From JBarata, unpublished data.

Appendix I

The work here developed was presented as poster communication at 1st ASPIC International Congress, Lisboa.

Prognostic significance of PI3K/Akt/mTOR pathway using phosphoflow in pediatric acute lymphoblastic leukemia – preliminary results

Mariana L. Oliveira[1], A. Margarida Gomes[1], Leila R. Martins[1], Joaquin Duarte[2] and João T. Barata[1]

[1] Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa;
[2] Serviço de Pediatria, Instituto Português de Oncologia Francisco Gentil, Lisboa (IPOL)

1 INTRODUCTION

Acute Lymphoblastic Leukemia (ALL)

The most frequent childhood malignancy.
Characterized by the accumulation of immature lymphoid cells within the bone marrow and lymphoid tissues.
Around 10-20% of patients relapse.

PI3K/Akt/mTOR pathway

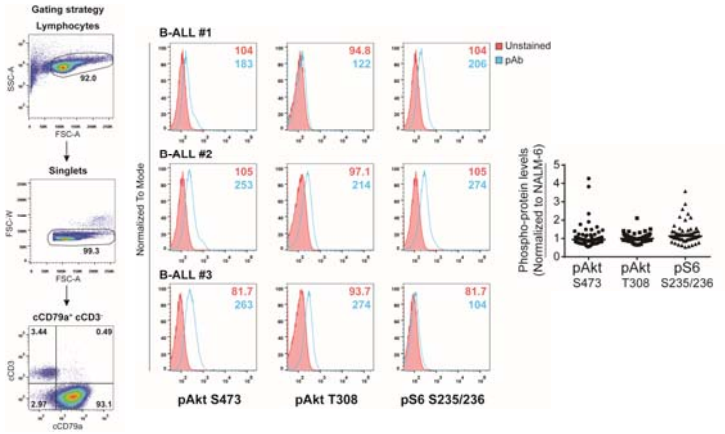
Pro-survival pathway extensively implicated in cancer and ALL.
Frequently hyperactivated in ALL, promoting leukemia cell viability.
Activated by cell-autonomous lesions and microenvironmental cues (IL-7).

Does activation of PI3K/Akt/mTOR pathway have prognostic value in pediatric ALL?

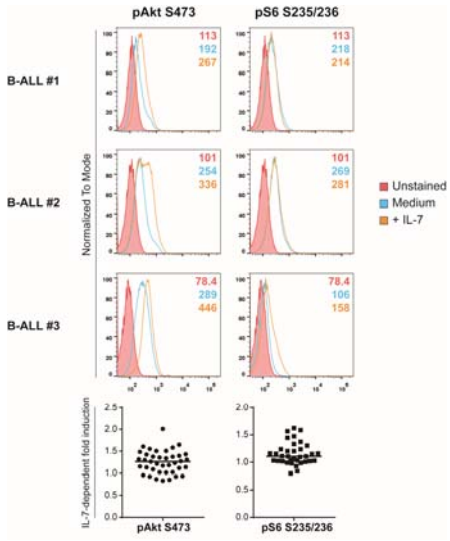
To answer this question, we used:

- ✓ Bone marrow samples (n=58) collected from pediatric ALL patients at diagnosis from the Pediatric Department of Lisbon IPOFG.
- ✓ A flow cytometry approach with phospho-specific antibodies (**phospho-flow**).

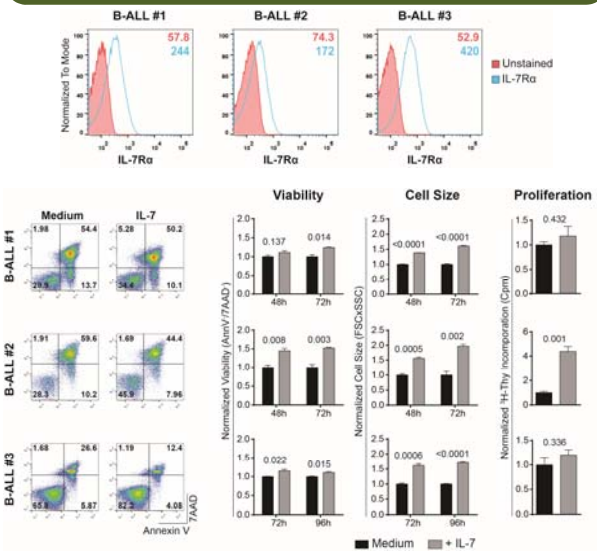
2 ALL samples display heterogeneous levels of basal PI3K/Akt/mTOR signaling pathway activation



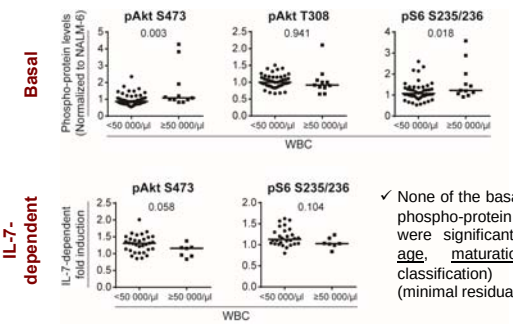
3 ALL samples display heterogeneous levels of PI3K/Akt/mTOR pathway activation in response to IL-7



4 High degree of heterogeneity of IL-7Rα expression and functional responses to IL-7 in ALL samples



5 Levels of Akt and S6 phosphorylation associate with WBC counts



✓ None of the basal or IL-7-stimulated phospho-protein levels analyzed were significantly associated with age, maturation stage (EGIL classification) or MRD status (minimal residual disease).

CONCLUSIONS

- ❑ Higher basal levels of S6 (S235/S236) and Akt (S473, but not T308) phosphorylation associate with higher WBC.
  - ➡ Two independent mechanisms leading to Akt activation in ALL.
- ❑ In contrast, the ability to respond to IL-7 may correlate with lower WBC.
- ❑ **Overall, our data suggest that there may be an association of high Akt (S473) and S6 (S235/S236) phosphorylation levels with high risk (*WBC counts*) but not with poor prognosis (*MRD status*).**
- ❑ Studies with further primary ALL samples are warranted.

This work was supported by a grant from Fundação para a Ciência e a Tecnologia (PTDC/SAU-ONC/122428/2010) to JTB. LRM received a postdoctoral fellowship from FCT. We thank the contribution of patients and their families, and the clinical director, physicians, and nurses from the Pediatrics Department of the Lisbon IPOFG involved in providing the primary samples for the studies presented here.